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(57) Abstract

The synthetic capacities of endosperm tissue are harnessed, for the production of an exogenous polypeptide, via the transformation of a cereal or other monocotyledonous plant with a genetic construct comprised of a structural sequence encoding the polypeptide and, upstream therefrom with respect to the direction of transcription, a segment containing at least one regulatory element that effects or regulates expression of the structural sequence in endosperm tissue. Downstream of the structural sequence, the genetic construct also includes a segment that contains a terminal-processing signal for completion of the processing of nascent mRNA.

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PRODUCING COMMERCIALLY VALUABLE POLYPEPTIDES WITH GENETICALLY TRANSFORMED ENDOSPERM TISSUE

Background of the Invention

The present invention relates to obtaining genetically transformed monocotyledonous (monocot) plants that produce seeds comprised of endosperm tissue expressing exogenous, polypeptide-encoding DNA. The present invention also relates to the use of such endosperm tissue to produce exogenous proteins, including biologically active substances like insulin, tissue plasminogen factor, and human growth hormone.

The endosperm of a monocotyledonous plant is comprised of the aleurone layer and the starchy endosperm. These endosperm tissues develop from a single triploid cell, which is the product of the fusion of a sperm cell nucleus and two egg cell nuclei, an event that is separate from the fusion that gives rise to embryo tissues. Aleurone cells form a layer that surrounds the starchy endosperm of seeds produced by monocot plants, including the agriculturally important cereals. During germination, the plant embryo secretes gibberellic acid (GA), a hormone that causes the aleurone layer to synthesize and secrete large amounts of a limited number

of hydrolytic enzymes. These enzymes degrade material, such as starch and protein, that is stored in the endosperm, resulting in the release of products used by the growing embryo.

For example, the aleurone layers from only ten half seeds of barley (cv. "Himalaya"), representing about a million aleurone cells, secrete more than 300 micrograms of the hydrolytic enzyme α-amylase in just 24 hours. Other cultivars used to produce malting for beer secrete three times this amount.

Despite this prodigious synthetic capacity, endosperm tissue has not been the object of extensive experimentation aimed at achieving expression of exogenous (foreign) DNA. Huttly and Baulcombe, J. Cell. Chem., Suppl. 12C (1988), at page 207, report GA-regulated expression in oat aleurone protoplasts into which they introduced a genetic construct consisting of a wheat α-amylase promoter and a DNA sequence coding for the enzyme β-glucuronidase. The expression was transient only, and no suggestion was made as to how or even why aleurone-specific expression of exogenous DNA at a level above single protoplasts should be achieved.

Summary of the Invention

It is therefore an object of the present invention to provide a method for producing valuable proteins by exploiting the synthetic capacity of aleurone tissue.

It is also an object of the present invention to provide a process for obtaining monocot seed containing a substance encoded by exogenous (foreign) DNA which is expressed by transformed endosperm.

It is yet another object of the present invention to provide a monocot plant that produces seeds comprised of

endosperm tissue genetically transformed to express an exogenous DNA sequence coding for a polypeptide.

It is still another object of the present invention to provide a plant having a stably heritable phenotype that is characterized by the presence of aleurone tissue producing a foreign (exogenous) polypeptide.

In accomplishing these objects, there has been provided, in accordance with one aspect of the present invention, a method for obtaining seed comprised of genetically transformed endosperm tissue, comprising the steps of (A) providing a genetic construct comprised of (i) a regulatory element which is expressed at high levels in an endosperm cell; (ii) at least one DNA sequence that encodes a polypeptide, which DNA sequence is under the transcriptional control of the regulatory element; and (iii) a terminal-processing signal positioned downstream of the DNA sequence with regard to direction of transcription, (B) injecting the genetic construct into a floral tiller of a cereal plant prior to anthesis in the plant; and thereafter (C) assaying seeds from the injected plant for the presence of an expression product of the DNA sequence in the endosperm of any of the seeds, thereby to identify a seed comprised of genetically transformed endosperm tissue. In a preferred embodiment, step (C) of the process comprises assaying for the expression product by using an antibody that recognizes the product. In another preferred embodiment, the DNA sequence of the genetic construct has a guanine and cytosine (G+C) content that is greater than 50%.

In accordance with another aspect of the present invention, a process has been provided for producing a polypeptide, comprising the steps of: (A) producing genetically transformed endosperm tissue that expresses a genetic construct as described above; and (B) isolating a polypeptide that is the product of such endosperm-based

expression. In one preferred embodiment, the aforesaid process comprises isolating exogenous protein from the endosperm of seeds obtained from a substantially uniform population of cereal plants. In another preferred embodiment, the process comprises isolating a polypeptide expression product of genetically transformed endosperm tissue from medium in which the tissue is cultured.

In accordance with yet another object of the present invention, there has been provided a differentiated monocotyledonous plant that produces seeds comprised of endosperm tissue, such as aleurone tissue, genetically transformed to express an exogenous DNA sequence which encodes a polypeptide. In a preferred embodiment, the plant is one of a substantially uniform population of monocotyledonous plants that produce seed comprised, respectively, of endosperm containing an exogenous protein. In another preferred embodiment, the exogenous DNA sequence codes for a polypeptide also encoded by a naturally-occurring gene but that has a guanine and cytosine (G+C) content that is higher than that of the naturally-occurring gene.

There has also been provided, in accordance with still another object of the present invention, seed of a monocotyledonous plant, e.g., wheat, barley, oats, sorghum, rye, millet, rice, maize, sugar cane and coconut palm, which seed contains endosperm comprised of an exogenous polypeptide.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of

the invention will become apparent to those skilled in the art from this detailed description.

Brief Description of the Drawings

Figure 1 is a schematic diagram illustrating the manufacture of a genetic construct suitable for use in accordance with the present invention.

Figure 2 is a representation of a Southern- blot hybridization analysis of DNA from control (untransformed) barley plants and plants transformed in accordance with the present invention.

Figure 3 is a representation of a gel-electrophoretic analysis of proteins secreted, respectively, by control barley plants and transformed plants within the present invention.

Figure 4A is a representation of a Western blot of stained proteins produced, respectively, by a control barley plant and transformed plants within the present invention; 4B is an autoradiograph of the same Western blot.

Figure 5 is a schematic diagram illustrating the manufacture of genetic constructs suitable for use with the GUS probe in the present invention. Figure 5A illustrates the construction of plasmid JR124; Figure 5B illustrates the construction of plasmid JR129; and Figure 5C illustrates the construction of plasmid JR133.

Figure 6 is a collage of two photographs depicting, respectively, fluorescence in the media (Fig. 6A) and in extracts (Fig. 6B) in a screening plate of barley seed ends obtained from tillers injected with the JR124 construct.

Figure 7 is a representative assay of tissue extracts from barley seed ends from tillers injected with the JR133 construct.

Figure 8A provides the results from hybridizing Southern blots of DNA from two JR124 construct transformants (124-2D2 and 124-2E4) with a probe derived from the GUS coding sequence. Figure 8B shows the results from different digests of DNA from plants transformed with the JR133 construct hybridized with the GUS coding sequence probe. Figure 8C presents the results of similar analyses utilizing different restriction enzymes for DNA from two of the plants (2G7 and 2G10).

Figure 9 presents the autoradiograph of a Southern blot of control barley DNA (C) and DNA for the F_2 generation progeny of the thaumatin transformants 10D1 and 12H2 hybridized with a thaumatin probe.

Figure 10 presents the autoradiograph of a Southern blot, demonstrating instability of constructs containing the GUS sequence in transformed plants.

Figure 11 presents a Southern blot autoradiograph that demonstrates that GUS sequences were lost from tissues taken from later growth of the JR124-2D2 plant, but were inherited by some of the progeny germinated from seeds of the first tiller of JR124-2D2.

Figure 12 presents the results of a Southern- blot analysis of hybridizing DNA in plants transformed according to the present invention. Figure 12A provides the methylation pattern of the 133-series GUS-positive parent DNA fragments in 2G6 compared with control DNA, as assessed by digestion with different enzymes. Figure 12B provides the results of a similar digestion of GUS-hybridizing DNA in 2G8, 4F1, and 4G1.

Figure 13A provides the Southern-blot hybridization results of the undigested major GUS-hybridizing sequences separated from bulk chromosomal DNA of 124-2D2#6 compared with those fragments resulting from digestion with EcoRI or HindIII. Figure 13B provides the electrophoretic

results of the undigested GUS-hybridizing fragments in progeny 2G7#3 and 5A7#8 compared with those fragments resulting from digestion with BamHI or EcoRI.

Figure 14 provides the results of methylation in the 2.5 kb HindIII-EcoRI fragment hybridizing to the thaumatin probe in DNA from the F_3 plant, G12-2-8, and the F_2 plant, 12H2-4, using MboI and MspI.

Figure 15 presents photographs depicting fluorescence in the media (Fig. 15A) and extracts (Fig. 15B) from a screening plate of oat seed ends obtained from tillers injected with the JR133 construct.

Detailed Description of Preferred Embodiments

Pursuant to the present invention, the synthetic capacities of endosperm tissue are harnessed, for production of an exogenous polypeptide, via the transformation of a cereal or other monocot plant with a genetic construct comprised of a DNA sequence (a "structural sequence") encoding the polypeptide and, upstream therefrom with respect to the direction of transcription, a DNA segment containing at least one regulatory element that effects or regulates expression of the structural sequence in endosperm tissue. Downstream of the structural sequence, the genetic construct also includes a DNA segment that contains a terminal-processing signal for completion of the processing of nascent mRNA.

More specifically, the phrase "terminal-processing signal" denotes a nucleotide sequence that is recognized, during post-transcription process of mRNA in vivo, as indicating where a precursor mRNA molecule should be cleaved to yield a mature mRNA species which will be translated. A segment containing a terminal-processing signal can be obtained by comparing cDNA encoding an

endosperm-expressed product, such as that for α-amylase, protease, protease inhibitor, or α-glucanase, with the genomic DNA coding for the same product, thereby to identify the 3′ terminus of the cDNA. By isolating a genomic DNA segment comprising 50 to 100 base-pairs on either side of the aforementioned 3′ terminus, one is assured of obtaining a segment with the terminal-processing signal.

Conventional techniques are available for making a <regulatory element/structural sequence/terminal-processing signal> fusion product, as described, e.g., by Schell, Science 237: 1176-83 (1987); Ellis et al., EMBO J. 6: 11-16 (1987) and Czernilofsky et al., DNA 5: 101-13 (1986). In this context, the phrase "regulatory element" denotes a nucleotide sequence that influences transcription of a structural sequence by influencing the movement of an RNA polymerase enzyme along the DNA template undergoing transcription. A preferred regulatory element, referred to here as a "promoter," includes (i) a site of initial recognition, prior to binding, between an RNA polymerase molecule and the DNA chain, (ii) a site for RNA polymerase binding and (iii) a site for initiation of transcription.

As indicated above, expression of a regulatory element suitable for the present invention should be especially strong in, or specific to, endosperm cells. Such a regulatory element can be obtained using cDNA produced from messenger RNA molecules (mRNAs) that are found exclusively in aleurone tissue, or at least are present at some stage of development or activation stage in endosperm tissue in amounts some 50-times or greater than corresponding amounts in either leaf and root tissues. Illustrative suitable promoters of this sort are the promoters for the low-pI α-amylase gene (Amy32b) as described by Rogers and Milliman, J. Biol. Chem. 259:

12234-12240 (1984), the high-pI α-amylase gene (Amy6-4) characterized by Khursheed and Rogers, J. Biol. Chem. 263: 18953-18960 (1988), and the promoter for a barley thiol protease gene ("Aleurain") as described by Whittier et al., Nucleic Acids Res. 15: 2515-2535 (1987).

More particularly, total mRNA can be isolated from endosperm cells, and poly(A) RNA selected and used to produce cDNA by reverse transcription, via known methodology described, for example, by Rogers, J. Biol. Chem. 260: 3731-38 (1985) (hereafter "Rogers (1985)"), the contents of which are hereby incorporated by reference. If the endosperm tissue is from the aleurone layer, the step of isolating total mRNA can optionally be preceded by a prolonged stimulation of the aleurone cells with GA, thereby to enhance the content of mRNA species transcribed under the control of a regulatory element that is GA-sensitive and, hence, presumably expressed in aleurone cells.

The cDNA thus produced is used to probe genomic DNA from a target monocot, such as a cereal crop (wheat, barley, oat, sorghum, rye, millet and rice), maize, sugar cane or coconut palm, in order to identify a structural sequence responsible for an abundant endosperm mRNA species represented in the cDNA. A segment of genomic DNA bordering the 5'-end of the structural sequence can then be isolated and incorporated into a genetic construct wherein the segment is fused to a structural sequence encoding a "marker" polypeptide which can be readily detected, e.g., using an antibody which recognizes an epitope presented by the polypeptide. A genomic-DNA segment of this sort that is on the order of 1,500 kilobases (kb) in length can be expected to include at least a promoter which can be employed as the regulatory element in the present invention.

The construct thus produced is used to transform a monocot plant so that detection of the marker can serve as an assay for the presence of a suitable regulatory element in a segment isolated, as described above, from genomic DNA. The same basic approach can also be used to introduce marker-encoding sequences into plants for varietal identification purposes, e.g., in the context of distinguishing malting barleys which have heretofore been differentiated only with difficulty.

More generally, the use of a non-antibiotic marker, as described herein, to screen plant transformants represents a departure from conventional practice, whereby transformants are screened on the basis of resistance to kanamycin or some other antibiotic. The latter approach requires expensive and complex facilities for the growth of large numbers of seedlings on precisely defined media into which the antibiotic is introduced. The high intrinsic resistance of cereals to antibiotics like kanamycin also means that the sensitivity of selection of transformants will be low, and that the false-positive rate will be high.

In conjunction with screening of transformants as described above, it is preferred that the transformants also be screened for the presence of methylation at adenine and cytosine bases. Transformants characterized by relatively extensive adenine methylation in the sequence GATC are less likely to maintain transforming DNA that is stably heritable. Accordingly, transformants are preferred in which the inserted DNA has undergone comparatively little methylation of adenines, but show some methylation of cytosine residues. These criteria of methylation pattern are readily assessed by using restriction enzymes with different sensitivities to methylation, such as MboI and Sau3AI, or MspI and HpaII.

A preferred means of transforming a monocot plant, pursuant to the present invention, entails injecting the construct into floral tillers of the target plant and then screening for transformed seeds produced by the injected tillers. In accordance with this approach, aliquots of an aqueous solution that contains the genetic construct are injected, prior to anthesis but after meiosis, into the hollow space above each developing inflorescence; total injected volume is typically around 300 μl. After injection in this manner, a floral tiller is allowed to grow to maturity and to produce seeds by self-fertilization or, in the event of self-incompatibility, by cross-pollination with other injected tillers. By this approach, transformation frequencies can be achieved that are sufficient to permit the development, via the selection method described herein, of genetically-modified plants which express exogenous DNA in endosperm tissue.

Whatever transformation technique is employed, seed from a putative transformant can be tested for the expression of foreign DNA by first separating the seed into an embryo-containing portion and a tip portion representing about 20% of the seed distal to the embryo. The latter portion is incubated in a suitable medium, which is thereafter tested, e.g., via an ELISA-type assay, to detect the presence of the polypeptide coded for by an exogenous structural sequence.

When the desired expression product is detected, the embryo-containing portion of the seed can be planted to obtain a differentiated monocot plant which can ultimately produce seeds comprised of endosperm tissue expressing the exogenous structural sequence. By conventional plant breeding techniques, the desired exogenous DNA sequence can be manifested in a homozygous condition and be capable of being passed on to subsequent

generations. In a preferred embodiment of the present invention, a plurality of such transformed seeds are planted to obtain a stand or population of plants, preferably cereal plants, that is substantially uniform to the extent that most or all of the plants in the stand produce seed containing the desired exogenous protein. The plants can be harvested, and the desired protein extracted from the seed, in the course of an otherwise ordinary agricultural operation.

Alternatively, transformed seed of the present invention can be used as the source of endosperm cells for culturing in a suitable medium, whence a desired polypeptide synthesized and excreted by the cultured cells can be extracted. For example, the technology for isolating and culturing endosperm tissue has long been available, as evidenced by Yomo and Varner, CURRENT TOPICS IN DEVELOPMENTAL BIOLOGY 111-44 (Academic Press 1971) (aleurone tissue) and 2 HANDBOOK OF PLANT CELL CULTURE Ch. 3 (Macmillan 1984) (starchy endosperm tissue).

The range of structural sequences that can be employed in the present invention encompasses, in addition to synthetic sequences, genes or portions of genes that encode products ordinarily made by plants. Typical of such products is the protein thaumatin, found in arils of the fruit of West African plant Thaumatococcus daniellii, which is the sweetest known substance and, hence, a commercially valuable food additive. By the same token, structural sequences that code for various bacterial and fungal proteases, themselves useful detergent components, are suitable for use according to the present invention.

More generally, structural sequences cloned from a variety of prokaryotic and eukaryotic sources are also suitable in this context. Exemplary of such cloned

sequences are those coding for hormones like insulin, bovine and human growth hormone, erythropoietin, atrial natriuretic factor, and the various colony stimulating factors (M-CSF, G-CSF, GM-CSF, interleukin-3, etc.); other growth and regulatory factors such as epidermal growth factor, insulin-like growth factor-1 and -2, nerve growth factor, transforming growth factor-α and -β and platelet-derived growth factor; the interferon proteins IFN-α and IFN-β; and proteins that are classified as monokines, such as interleukin-1α, interleukin-1β and tissue necrosis factor, or lymphokines, like interleukin-2 and IFN-γ.

It is preferred that the exogenous, polypeptide-encoding DNA used, according to the present invention, to produce transformed aleurone tissue should be rich in the bases guanine (G) and cytosine (C), in the sense that the (G+C) content of the DNA is higher than 50%, and preferably in the range of 60% to 65%, as determined from the DNA sequence. The class of structural DNAs satisfying this requirement includes virtually all studied cDNAs and genomic clones representing naturally-occurring genes expressed in aleurone tissue. See Khursheed and Rogers, J. Biol. Chem. 263: 18953-18960 (1988); Whittier et al., Nucleic Acids Res. 15: 2515-2535 (1987). For other eukaryotic and prokaryotic DNAs that are not (G+C)-rich in native form, synthetic or mutant DNA segments can be obtained that code for the polypeptide of a naturally-occurring gene but that are modified in such a manner that the (G+C) content is increased above 50%, and more preferably to 60%-65%.

The polypeptide-encoding DNA segment sequence can be modified, for example, using a computer program like "Codon Preference," available from the University of Wisconsin Genetics Computer Group [see Devereaux et al., Nucleic Acids Res. 12: 387-395 (1984)], to have increased

(G+C) content while still encoding the same polypeptide. Once a desired polypeptide-encoding DNA sequence is determined, the encoding sequence can be synthesized by mutation of the cloned cDNA or genomic DNA [see, e.g., CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Chapter 8 (Ausebel, et al., eds. 1989)] or by synthesis via ligation of long (>100 base pairs) synthetic oligonucleotides. See Ausebel et al., supra, at pages 8.2.8 to 8.2.13.

It is also preferred that the exogenous DNA employed in transforming aleurone tissue pursuant to the present invention should not methylated at adenosine bases. For purposes of producing usable amounts of exogenous DNA, therefore, a cloning system that does not methylate adenine is preferably employed. For example, all enteric bacteria possess the so-called dam restriction/methylation enzyme that methylates adenine at all GATC sites; it is for this reason that all plasmids grown in wild-type E. coli strains have every GATC site methylated. Dam⁻ E. coli strains are readily available commercially, for example, from Stratagene, La Jolla, CA (strain GM48), to use in cloning exogenous DNA for the present invention.

The present invention is further described below with regard to the following illustrative examples.

EXAMPLE 1. PRODUCTION OF GENETIC CONSTRUCTS

To form DNA coding for a marker polypeptide, the nucleotide sequence encoding prothaumatin protein, minus the signal peptide (i.e., the portion involved in the transport of the protein into the rough endoplasmic reticulum) and first seven amino acids of the mature protein, was fused in frame at the codon for the eighth thaumatin amino acid to a sequence coding for the signal

peptide portion and first seven amino acids of the so-called "probable amylase/protease inhibitor" (PAPI) barley protein described by Mundy and Rogers, Planta 169: 51-63 (1986) (hereafter "Mundy & Rogers (1986)").

Prothaumatin cDNA was obtained for this purpose from a plasmid, pUR528, produced by Edens et al., Gene 18: 1-12 (1982) (hereafter "Edens (1982)"), the contents of which are hereby incorporated by reference. Prothaumatin-encoding DNA can be synthesized, using conventional methodology, from the nucleotide sequence disclosed by Edens (1982). Alternatively, pUR528 can be obtained for research purposes from Unilever Research Laboratories (Vlaardingen, the Netherlands).

Figure 1 depicts the approach used in producing genetic constructs that incorporate the above-mentioned marker sequence (designated "JR073") bracketed, at the 5' end, by a segment of cereal (barley) genomic DNA taken upstream from a known α-amylase structural sequence and, at the 3' end, by another genomic DNA segment containing a terminal-processing signal for the same α-amylase sequence. For each construct, the segment containing the regulatory element was about 1.5 kb in length, while the segment comprising the terminal-processing signal was about 215 bp.

As shown in Figure 1, illustrative construct No. JR083 incorporated a segment containing a promoter ("Amy32b promoter"), which segment had been positioned just upstream of a known barley α-amylase gene described by Rogers and Milliman, J. Biol. Chem. 259: 12234-12240 (1984). Construct No. JR117 (not shown) included construct No. JR083 and additional exogenous DNA included for experimental purposes.

EXAMPLE 2. OBTAINING GENETICALLY-MODIFIED MONOCOT PLANTS AND SCREENING SEEDS FOR TRANSFORMED ALEURONE TISSUE

Injection of Exogenous DNA and Analysis of Transformants: A solution of plasmid DNA (100 μg/ml in distilled water; total volume of 300 μl) produced in accordance with Figure 1 was injected just above a developing floral node when that structure was at or just above the third leaf in a tiller with five leaves. Based on morphological criteria, the corresponding developmental time was adjudged to be about two weeks before anthesis.

Seeds produced by injected tillers were harvested and placed individually into wells in a 96-well microtiter dish. To screen for production of thaumatin, the tip of each seed opposite the embryo (about 1/5 the volume of the seed) was cut off and placed in an identical position in a duplicate dish. The remaining 4/5 of the seed was stored at 4½C for use, as needed, for germination and growth of a plant.

The smaller fragments were sterilized by treatment with 70% ethanol (1 minute) and with 0.2% silver nitrate solution (20 minutes), respectively, and then allowed to air dry for 30 minutes. To each well was added 100 μl of incubation buffer (20 mM sodium succinate (pH 5.2), 10 mM $CaCl_2$, 10^{-6} M GA, 100 μM leupeptin, 50 μg/ml carbenicillin and 125 μg/ml Fungizone®, an antibiotic to inhibit fungal growth). Under these incubation conditions, the aleurone layers were activated and vigorously secreted α-amylase.

Incubation at room temperature, in a humidified atmosphere, continued for two or three days (the time of incubation differed in different experiments and was not critical for the outcome). Thereafter, 50-μl aliquots from each well were transferred onto a replica of the microtiter plate made by sandwiching nitrocellulose membrane (Schleicher & Schuell, New Hampshire), wet with

distilled water, in a 96-well "dot blot" apparatus (Bethesda Research Laboratory). After all samples were filtered through the nitrocellulose, the individual wells were washed with 150 μl of TBS.

Pursuant to the methodology of Mundy & Rogers (1986), the resulting Western dot blots were subjected to an enzyme-linked assay (ELISA) for material recognized by antithaumatin antiserum (see below). Color reactions were allowed to proceed so that negative controls still had no color background or a very faint background.

To produce rabbit antithaumatin antibodies for use in Western dot-blot screening, a mixture of thaumatins I and II, referred to hereafter as "thaumatin," was purchased from Sigma Chemical Company (Cat. No. T7638) and cross-linked with glutaraldehyde, in accordance with Bollum, Proc. Nat'l Acad. Sci. USA 72: 4119-122 (1975). Thaumatin I and thaumatin II differ at five amino acid positions and have slightly different pI's.

More specifically, 3 mg of rabbit albumin were dissolved in 0.4 ml of 0.15 M NaCl/0.05 M Tris-HCl solution (TBS; pH 7.9). To this were added 40 μl of a 10 mg/ml solution of thaumatin in distilled water, followed with 100 μl of 21 mM glutaraldehyde (Sigma Chemical Co.) added dropwise over a total time period of one hour. The tube was allowed to incubate at room temperature overnight, and then the cross-linked proteins were dialyzed against 1 l of TBS at 4½C for six hours.

A volume of the protein solution containing 100 μg was then emulsified with an equal volume of complete Freund's adjuvant and injected at multiple sites subcutaneously in a New Zealand White rabbit. The rabbit was boosted with antigen in incomplete Freund's adjuvant at two week intervals until an adequate antibody titer was obtained. In initial experiments, the antiserum obtained was used without further purification in

screening for transformed seeds. Later, to minimize nonspecific background in Western blot analyses of proteins produced by transformed plants, antithaumatin antibodies were affinity-purified.

This purification was carried out on a thaumatin-sepharose affinity column carrying 5 mg thaumatin/ml cyanogen bromide-activated Sepharose 4B (Sigma Chemical Co.), and coupling of protein was effected according to the manufacturer's direction. Rabbit serum was heat-inactivated at 56½C for fifteen minutes, cooled on ice, and then passed through the column at room temperature. The column was washed with TBS until the A_{280} of the effluent dropped to background. Specifically, adsorbed immunoglobulins were eluted with 0.2 M glycine/HCl (pH 2.2), dialyzed against TBS, and then stored in aliquots at -20°C.

Seed remnants were planted that corresponded to the foregoing test dots that appeared to be above background levels. When the plants grown from these remnants were large enough to tolerate removal of about 0.5-1 g of leaf tissue, that portion was removed and genomic DNA was isolated, pursuant to the methodology of Dellaporta et al. in MOLECULAR BIOLOGY OF PLANTS: A LABORATORY COURSE MANUAL 36-37 (Cold Spring Harbor Laboratory, 1984). The isolated DNA was then digested with restriction enzymes, electrophoresed, and transferred to Zetaprobe® nylon membrane (Biorad) for Southern-blot analysis, pursuant to Whittier et al., Nucleic Acids Res. 15: 2515-35 (1987), with a probe derived from the thaumatin-coding cDNA of Edens (1982). The stringency of the wash conditions, 0.1 x SSC - 0.1% SDS at 65½C, was high, and control DNA did not hybridize to the probe. The results of the screening and Southern blotting are shown in Table 1 below.

Table 1

Construct	No. of seeds screened	No. of seeds planted	No. of viable seeds	No. positive Southern blot
083	576	16	13	1
	480	8	7	5
117	96	7	6	3

A pair of representative Southern blots is presented in Figure 2; these are from gels run in parallel. In Figures 2A and 2B, the DNAs were digested with HindIII and EcoRI, restriction enzymes that cut the intact construct such that the promoter-gene-terminator sequences (about 2.4 kb) are freed from surrounding DNA. In Figure 2C and 2D, the DNAs were digested with NotI and EcoRI; these should give a different pattern depending upon the relative position of sites accessible to these enzymes in the plant DNA.

The blots were first hybridized with the thaumatin coding-sequence probe (Fig. 2A and 2C). After the membranes were washed extensively at 65°C in 0.015 M NaCl/0.00015 M sodium citrate/0.1% SDS solution, they were exposed for twenty-four hours at -80°C with an intensifying screen. The membranes were heated (100°C in the aforementioned buffer for fifteen minutes), and thereafter exposed for three days with an intensifying screen, to ensure that all probe was removed.

The membranes were then rehybridized (Figs. 2B and 2D) with a probe derived from a low-pI α-amylase cDNA corresponding to "clone E" described by Rogers and Milliman, J. Biol. Chem. 258: 8169-74 (1983). This cDNA is approximately 93% identical to the low-pI α-amylase genomic clone containing the amy32b promoter, and the

sequence similarity is highly conserved in the 3' untranslated region. Therefore, if the genetic construct introduced into the transformed plant chromosome was intact, a restriction fragment generated from the plant DNA, using the above-mentioned enzymes, should hybridize both to the thaumatin probe and to the "clone E" cDNA probe.

The DNAs used on the blots were from: [1] plant "G12," which was a 117-series transformant and the parent plant (p) of four progeny plants that germinated from G12 seed (of ten planted); [2] two plants, designated "10D1" and "12H2," from the 083-series transformants; and [3] from a plant (C) that was grown alongside the others, but was not transformed. In every instance, DNA from the control plants did not hybridize to the thaumatin probe, although the expected hybridization of endogenous amylase genes with the "clone E" probe was observed, demonstrating that adequate amounts of DNA were indeed present on the filter.

The G12 parent and two progeny plants, Nos. 2 and 7, and plants 10D1 and 12H2 all yielded DNA with fragments that hybridized both to the thaumatin and the clone E probes. Thus, it was understood that these plants were transformants, and that the exogenous DNA was heritable to the second generation.

Seeds were tested from two 083-series transformants, 10D1 and 12H2, and from an F_2 generation plant ("G12-2") from the 117-series plant G12. For each plant, eighty-five aleurone layers, plus layers from a nontransformed control, were labeled for twenty-four hours with [^{35}S]methionine, and aleurone proteins which were antigenically related to thaumatin were selected from the culture media on individual antithaumatin-sepharose affinity columns.

These proteins were analyzed sequentially in two different electrophoretic systems. First, the proteins were loaded onto an acid-urea gel in the order: 5μg thaumatin marker (T), control proteins, T, 10D1, T, G12-2, T, 12H2, T, T. When the pyronin Y marker dye reached the bottom of the gel, the gel was cut vertically between the right-most two T marker lanes. The single T marker lane was electroblotted onto polyvinyldifluoride (PVDF) membrane (Immobilon®; product of Millipore Corp.) and stained with Coomassie Blue to identify the position of that protein. The appearance of that marker lane is presented in Figure 3, left portion, where the orientation corresponding to the direction of electrophoresis is top (+) to bottom (-). The thaumatin marker (Sigma Chemical Co.) always contained three different species of proteins, with the major component electrophoresing ahead of two minor species.

The remaining portion of the gel was cut into two portions, indicated by (a) and (b), where (a) included 1 cm containing the trailing portion of the main marker band and the two minor components; (b) included 1 cm containing the main marker band and the portion of the gel immediately in front of it. Each of these strips was then individually equilibrated with the proper buffer and placed into a long horizontal well of a standard sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), and the proteins within the strip were electrophoresed, in the (-) to (+) direction, to separate them according to molecular mass.

On either side of the strips, in separate wells, were loaded thaumatin marker (left) and a mixture of 5 other marker proteins of differing molecular masses (right). (The numbers on either side of the panels indicate the molecular mass of these markers in kilodaltons.) When the marker dye reached the bottom of

the gel, electrophoresis was terminated and the gel contents were electroblotted onto a PVDF membrane. The proteins were stained with Coomassie Blue, and then the newly synthesized proteins were identified by autoradiography.

Each of Figures 3(a) and 3(b) is a representation of the stained membrane (top) and its autoradiograph (below). The positions of the marker proteins are indicated for the autoradiographs; these positions were confirmed by cutting out the appropriate bands from the membranes and re-exposing to x-ray film (not presented). In (a), it can be seen that the two minor thaumatin marker bands migrating more anodally in the acetic acid-urea gel are indistinguishable in size to the bulk of thaumatin (22 kd) on SDS-PAGE, since the only stained protein band in those lanes (indicated by "t" above each) migrates with that marker. This result indicates that the bands represented species of thaumatin with different overall charges but with substantially similar molecular masses, a result to be expected in light of the previously mentioned fact that "thaumatin" is a mixture of slightly different forms.

The autoradiograph demonstrates that the 12H2 sample contained a labeled protein that electrophoresed in the same manner as did the thaumatin markers (arrow). This protein (approx. 22 kd) was not present in control (C), 10D1, or G12-2 samples.

Further evidence for the specificity of this ~22 kd 12H2 protein was obtained by analyzing proteins synthesized in aleurone tissue but not secreted. Thaumatin is a storage protein and might be expected to accumulate in protein bodies in aleurone cells. Accordingly, aleurone layers from two hundred de-embryonated half-seeds from a control plant (C) and from 12H2, respectively, were incubated in the presence of

[^{35}S]methionine and GA for forty-eight hours. After harvesting the media, the aleurone layers were washed with distilled water and then homogenized with a tissue homogenizer in 20 ml of TBS solution (0.15 M NaCl/0.05 M Tris-HCl; pH 7.9) containing 1 mM phenylmethylsulfonyl fluoride, 100 μM leupeptin and 2% insoluble polyvinylpyrrolidone.

The samples were diluted to 50 ml with TBS and made 0.1% with respect to NP40. Insoluble debris was removed by centrifugation in a desk centrifuge for ten minutes, and to each supernate was added 0.25 ml of 20% NP40, a detergent product sold by Shell Oil Co. The supernates were each incubated overnight, at 4½C on a rotating shaker, with 1.5 ml of antithaumatin-sepharose, 1 ml of which contains about 9 mg of rabbit immunoglobulin selected by adsorption to a thaumatin-sepharose affinity column. After an extensive washing with TBS - 0.1% NP40, proteins specifically adherent to the column were eluted with 0.2 M glycine - HCl (pH 2.2) and then precipitated with 10% trichloroacetic acid. Proteins were similarly selected from the media samples.

The precipitated protein samples were dissolved in sample buffer and loaded onto an acetic acid-urea gel in two sets: in one set, 20% of each sample was loaded sequentially, followed by two consecutive wells containing 5 μg each of thaumatin marker; the second set, separated by empty wells on either side from thaumatin markers was made up of the remaining 80% of each sample. After electrophoresis, the gel was cut vertically between the two center thaumatin marker lanes. The portion of the gel containing the first set of samples was electroblotted onto PVDF membrane and stained to visualize the transferred proteins.

A representation of this blot, with its autoradiograph, is presented in Figure 4A. The remaining

half of the gel was cut horizontally into two strips: strip 'd' was 0.5 cm wide and included the trailing portion of the thaumatin marker; strip 'c' included the 0.5 cm of gel immediately to the anodal side of strip 'd'. These two strips of gel included two prominent stained protein bands in the 12H2 extract sample lane that are not visualized in the other sample lanes.

The two gel strips, 'c' and 'd', were equilibrated with SDS-PAGE buffer and inserted horizontally, side-by-side, into a long well of a 2 mm-thick gel for SDS-PAGE analysis. Strip 'c' was cut to include only the four lanes containing the barley proteins. Strip 'd' included the portions of the gel with thaumatin marker protein bracketing the four lanes with barley proteins. On either side of the long well containing the gel strips were single wells loaded with thaumatin marker protein (left), and a protein marker mixture (right).

After electrophoresis, the proteins from the SDS--PAGE gel were electroblotted onto PVDF membrane and visualized by protein stain and by autoradiography (Fig. 4B). It was observed that the lane from the 12H2 extract in both strips contained proteins that resolved into multiple bands on SDS-PAGE. In strip 'd', the major protein band visualized by stain (arrow) electrophoresed identically to the internal thaumatin marker (t); on the autoradiograph, this protein band was labeled with [^{35}S]met. Upon prolonged exposure with fluorography, a faint band of identical size was visualized in the 12H2 medium lane, but not in the control lanes; this result was consistent with the observations described in relation to Figure 3. All lanes from this strip had a labeled protein (>16 kd) in roughly equal amounts, demonstrating that the absence of other labeled proteins in the control lanes was not due to inadequate sample

loading. The 12H2 extract lane in strip 'c' also contained a labeled protein that electrophoresed identically to the thaumatin marker (arrow).

These results demonstrate that a plant, 12H2, carrying an apparently intact recombinant gene, produced seeds that synthesized a protein in their aleurone layers that was very similar in charge to (as assessed on acetic acid-urea gel electrophoresis) and indistinguishable in size (as assessed on SDS-PAGE) from thaumatin. The observed expression in aleurone tissue was what would be predicted from the amylase promoter sequences fused to the recombinant thaumatin structural sequence. The protein encoded by the latter sequence was recognized by antithaumatin antibodies and was not present in extracts from control aleurone layers. A newly synthesized protein with these same characteristics was also secreted from 12H2 aleurone layers.

EXAMPLE 3. USE OF E. COLI β-GLUCURONIDASE (GUS) AS A MARKER TO DETECT TRANSFORMED PLANTS

Production of Recombinant DNA Segments Wherein the GUS Coding Sequence is Under Transcriptional Control of a Known Aleurone Promoter Sequence: Construction of plasmids JR124, JR129, and JR133 is schematically presented in Figure 5. For the JR124 construct (Fig. 5A), the coding sequence of E. coli β-glucuronidase (GUS) was bracketed by the Amy32b promoter/upstream sequences and the Amy32b 3′ sequences for RNA processing/poly(A) addition. For the JR129 construct (Fig. 5B), the promoter/upstream sequences and part of the coding sequence for the barley gene Aleurain were fused to the GUS coding sequence. For the JR133 construct (Fig. 5C), the GUS coding sequence was fused to the N-terminal coding sequence of the barley α-amylase gene, Amy6-4.

The completed construct brackets the fused coding sequence between the Amy6-4 upstream/promoter region and the Amy6-4 3′ sequences for RNA processing/poly(A) addition. An example of the use of GUS in the Agrobacterium-mediated transformation of dicot plants has been described in Jefferson et al., EMBO J. 13: 3901-3907 (1987).

Transformation of Cerial with GUS Construct: Barley tillers were injected with purified plasmid DNA (100 μg/ml in distilled water) as described in EXAMPLE 2.

Screening for GUS Activity: Seeds were prepared for screening essentially as described in EXAMPLE 2. Grain to be screened was cut with a razor blade to remove ca. 1/5 of the grain end opposite the plant embryo. The seed end was then placed in a well of a 96-well microtiter dish. The remaining portion of each seed containing the embryo was placed in an identical position in a duplicate dish for later use. When initiating screening, grain ends were first sterilized by washing in 70% EtOH for 1 minute, then in 0.2% $AgNO_3$ for 20 minutes, by adding the solutions to microtiter dish wells and aspirating as appropriate. The seed ends were subsequently allowed to completely dry in a sterile tissue culture hood to ensure that fungal spores are killed.

To each well containing sterile seed ends, 100 μl of incubation buffer (20 mM sodium succinate (pH 5.2), 10 mM $CaCl_2$ containing 10^{-6} M GA_3, 50 μg/ml carbenicillin, and 120 μg/ml amphotericin B (Fungizone®)) was added. Incubation of the sterile seed ends and a row of ends (12) from control seeds continued for two days at room temperature in a humidified chamber.

For assaying GUS activity, 4-methyl-umbelliferyl-β-D-glucuronide (MUG) was added, to a final concentration

of 1 mM, to the incubated media samples. The samples were then allowed to incubate overnight at room temperature in a humidified atmosphere to prevent evaporation. Subsequently, media samples were removed and transferred to identically-positioned wells in a fresh microtiter dish. To the media in each well, 5 μl of 2 N NaOH was added and mixed gently. The resulting media was placed on a long-wavelength (>300 nm) UV light and positives exhibited a blue fluorescence. Photographs were taken using a Wratten #3 filter.

To each well was added 25 μl of homogenization buffer (50 mM $NaPO_4$ (pH 8), 10 mM EDTA, 0.1% NP40, 0.1% sarkosyl, and 1 mM phenyl methyl sulfonyl fluoride (PMSF) containing 1 mM MUG). Each seed portion was then thoroughly ground with a teflon pestle to ensure separation of the cells of the aleurone layer. Additional homogenization buffer (100-150 μl) was added to each well and the samples were incubated overnight at room temperature. Subsequently, 50 μl of media were removed from each well, transferred to an identically-positioned well in a new microtiter dish, supplemented with NaOH as above, and viewed under UV light.

To confirm possible positives, the remainder of each grain was imbibed overnight, planted, and allowed to grow until the plant was large enough to produce sufficient leaf DNA for Southern-blot analyses as described in EXAMPLE 2.

Representative Examples of Positive REsults from GUS Screening Assays: Figure 6 depicts the media (Fig. 6A) and extracts (Fig. 6B) from screening plate #2 of seeds from tillers injected with JR124. The results demonstrate that well D2 fluoresces the brightest in both media and extracts, while well E4 may also give a positive result. The variable amounts of starchy

material carried over during the transfer process from the enzyme incubation plate to the plate for alkanization and viewing can account for the light-appearing wells in the plate with extracts (Fig. 6B). These light-appearing wells are easily distinguished from the blue fluorescence in wells D2 and E4 (identified in subsequent figures as 124-2D2 and 2E4, respectively).

Seeds from tillers injected with construct JR129 were screened at the same time. Fluorescence for the positive seed 129-6B3 in media and extract samples was similarly identified, but was less intense than that of the 124 positives (124-2D2 and 2E4).

Figure 7 presents results from assaying barley seed ends from tillers injected with construct JR133 for tissue extracts only (the media from the seed end incubations was not screened). Wells G6, G7, G8, and G10 (referred to in subsequent figures as 133-2G6, 2G7, 2G8, and 2G10, respectively) exhibited strong fluorescence as seen in comparison with wells containing extracts from control seeds (E1-7) and empty wells (F5-12).

Evidence That Positive-Screening Plants Carry the Inserted Construct: Figure 8A provides the results from hybridizing Southern blots of DNA from two JR124 construct transformants 124-2D2 and 124-2E4 (A), with a probe derived from the coding sequence of GUS. DNA from the two transformed plants was digested with BamHI, with EcoRI, or with a combination of both enzymes. Electrophoresis, transfer to a nylon membrane, and hybridization were performed as described above. The results indicate that the GUS probe hybridized to a ~6.5 kb EcoRI fragment in each DNA preparation. When the DNAs were digested with a combination of enzymes, the probe hybridized to a ~2.1 kb fragment (arrow) in each transformed plant; this is what was expected from the

original construct if it were intact in the barley chromosomal DNA. For the DNA digestions with BamHI, two fragments in the 2D2 plant hybridize (~12 kb and ~2.5 kb), while for plant 2E4, only one (~6.5 kb) fragment was identified. This finding is consistent with different positions and/or different final arrangements of the inserted genes in the barley chromosome. DNA from control plants did not hybridize to the GUS probe in this manner.

In a similar experiment conducted with DNA derived from the plant (well 6B3) transformed with DNA from the JR129 construct, the GUS probe identified a ~6.5 kb fragment in both BamHI and EcoRI digests, and a ~2.1 kb fragment in the DNA digested with a combination of the restriction enzymes. These results are consistent with the structure of the original transforming construct.

On the basis of the GUS screening assay, seven putative positive transformed seeds (2G6, 2G7, 2G8, 2G10, 4F1, 4G1, and 5A7) were germinated and tested for the presence of the inserted gene by Southern-blot analysis. Figure 8B shows the results from different digests of the JR133 construct hybridized with the same GUS coding sequence probe. Figure 8B demonstrates that all the different DNAs that were digested with a combination of HindIII and XhoI gave a hybridizing fragment to the GUS probe of ~7.3 kb whereas the control DNA did not have this fragment. An identically sized fragment was generated by HindIII alone; whereas XhoI does not cut barley DNA prepared by this technique.

To ensure that other restriction enzyme sites expected for this construct were present, DNA from two additional seeds (2G7 and 2G10) was further characterized (Fig. 8C). These results demonstrate that the enzyme BclI also cut the DNA preparations poorly; the GUS probe hybridized to a high molecular weight smear of uncut DNA

in each preparation treated with BclI. This indicates that the transforming DNA is integrated into the large barley chromosomal DNA and does not represent contaminating plasmid DNA which would have migrated further into the gel producing a discreet band.

Digestion of DNA from 2G7 with the restriction enzyme EcoRV produced four closely spaced fragments. This result, in combination with the results in Figure 8C from digestion with HindIII and XhoI, suggests that the transforming DNA formed tandemly arranged repeats of itself before integrating into the barley chromosomal DNA. This result has been well-documented for DNA transfected into tobacco protoplasts. See, e.g., Czernilofsky et al., DNA 5: 101-113 (1986).

In contrast to the results from the digestion of DNA from 2G7, digestion of DNA from isolate 2G10 with the same restriction enzyme (EcoRV) produced only a single hybridizing band. Further, another difference is that the combination digest with HindIII and BclI for 2G7 produced a result consistent with the inability of BclI to cut most sites in barley DNA, whereas the combination digest for 2G10 produced a smaller fragment, ~2.2 kb, as well as a fragment slightly larger than 7.3 kb. These results conform to what would be expected for different transformants where the foreign DNA was integrated into different places in the chromosomal DNA.

EXAMPLE 4. STABLE INHERITANCE OF PHENOTYPE ENCODED BY A (G+C)-RICH, ALEURONE-TRANSFORMING CONSTRUCT

Figure 9 presents the autoradiograph of a Southern blot of control barley DNA (C) for the F_2 generation progeny of the thaumatin transformants 10D1 and 12H2. [All progeny utilized in inheritance experiments are products of self-pollination.] The blot was hybridized

with a thaumatin probe. The results demonstrate that the 10D1 and 12H2 progeny have a 2.5 kb hybridizing band diagnostic of the thaumatin transformation marker which is lacking in the control, indicating that the thaumatin sequence is stably inherited. Further, tests have been done that clearly show stable inheritance for as far as the third in-bred generation (two generations each for 10D1 and 12H2, and three generations for the G12 plants described in EXAMPLE 2).

EXAMPLE 5. LACK OF STABLE INHERITANCE OF PHENOTYPE ENCODED BY AN (A+T)-RICH, ALEURONE-TRANSFORMING CONSTRUCT.

Original DNA preparations were made from the "parent" GUS transformants, that is, from the primary transformants. Parent DNAs were prepared when the plants had two tillers and when the smaller tiller was estimated to be about 2 grams. At this stage, the second tiller (smallest) was then removed and used for DNA preparation. When the first seeds were mature, 12 seeds from each transformant 124-2D2 and 133-2G7, -2G8, -2G10, -5A7, were planted. These seeds were all derived from the first (largest) tiller that developed because they were the first to mature. Additionally, more DNA from the parents was subsequently prepared in anticipation of progeny-parent blot comparisons.

JR133 Transformants: All DNA from plants that germinated were analyzed. Only one seed from 2G8 germinated, and the resultant plant was negative (data not presented).

All 2G10 progeny, and the repeat preparation of parent DNA were negative. The original 2G8 preparation was compared with a subsequent preparation. In addition, the blot also contained two preparations of 2G10 parent DNA

(Fig. 10). This figure shows that DNA first prepared from the 2G8 parent has a ~7.3 kb GUS-hybridizing band, while the other 3 samples were negative. Similar analyses were performed on 2G7 and 5A7 parent and progeny DNAs. None of the 2G7 or 5A7 progeny DNAs had a ~7.3 kb hybridizing band, but 2G7#3 and 5A7#8 each had a small, ~0.5-0.7 kb fragment that hybridized strongly to GUS. Control DNA from a plant grown alongside the test plants was included in all blot analyses and was always negative.

JR124-2D2 Pedigree: Consistent with previous results, the parent DNA isolated from tissue developing later lacked hybridizing sequences (Fig. 11). Progeny numbers 4, 6, and 7 had bands on a BamHI-EcoRI digest that hybridized to GUS; for progeny numbers 3 and 5, the strongest bands were the expected 2.1 kb size, while progeny 6 and 7 also had large hybridizing fragments. The intensity of the 2.1 kb bands was greater than would have been expected for single copy sequences.

These results demonstrate that the original transforming DNA identified in the parent plants was lost during plant development. This loss may have occurred in meristematic cells (regions of active mitosis and cell division) where the tissue was negative when mature. In the 133 series, small remnants of the original transforming DNA was identified in two progeny, but no intact constructs were present. For 124-2D2, the DNA was also lost from the parent, but some progeny retained large fragments that hybridized to GUS. All progeny exhibited evidence of gene rearrangement, and two progeny appeared to have multiple copies of the marker gene.

JR129 Transformants: GUS marker DNA was also lost from the parent, and ten progeny tested were all negative.

Analysis of Hybridizing DNA in Unstably Transformed Plants: In order to confirm the stability results, the structure and methylation patterns of hybridizing DNA were analyzed in the 133-series parents that were GUS-positive and in the 124-2D2#6 DNA which appeared to have multiple copies. These methylation patterns were then compared to those occurring in F_2 and F_3 generation plants that were positive for the thaumatin marker. Additionally, the structures of the small hybridizing fragments identified in 2G7#3 and 5A7#8 were analyzed.

First, the methylation pattern of the 133-series GUS-positive parent DNA fragments in 2G6 was determined and then compared to an equal amount of control DNA spiked with ~3 copies per haploid genome of JR133 plasmid DNA (Fig. 12). Restriction enzymes that have the same recognition site (so-called isoschizomers), but that have different responses to methylation of residues in that site were used. For 2G6 (Fig. 12A), HindIII produced a ~7.3 kb band; the band remained the same size following digestion with HindIII plus MboI. In contrast, the MboI isoschizomer, Sau3AI gave the expected 500 bp hybridizing fragment. A combination digest with Sau3AI and HpaII (lane 4) or MspI (lane 5) cleaved the Sau3AI fragment to much smaller fragments. The results in Figure 12A indicate that the hybridizing sequences in 2G6 are methylated at adenine residues that would inhibit MboI, but are not methylated at cytosine residues that would inhibit HpaII and MspI.

GUS-hybridizing DNA in undigested 2G6, 4F1, and 4G1 remained with high molecular DNA at the top of the gel

(not shown). This result is consistent with data presented in Figure 8C for 2G7 and 2G10. Figure 12B demonstrates that MboI releases the GUS-hybridizing fragments in 2G8, 4F1, and 4G1 to give ~15 kb and ~7 kb fragments in each (lanes 1, 4, 7). The combination of MboI and MspI (lanes 2, 5, 8), or MboI and HpaII (lanes 3, 6, 9), results in loss of the large fragments with the generation of ~200 bp hybridizing fragments in each, but 4F1 has a discreet ~1 kb hybridizing fragment (lanes 5 and 6) indicating that some cytosine residues were methylated.

These data indicated that all 133 parent DNAs tested had the marker DNA present in a high molecular-weight form that could not be separated from chromosomal DNA by gel electrophoresis when uncut. Further, the N-6 methyladenine sensitive enzymes, MboI and BclI, cut only a limited number of sites (presumably because the others were methylated), and there was a defined topography of methylation surrounding the marker sequences because MboI released the markers from high molecular weight, giving two discreet fragments in each instance. Additionally, the fact that the 7.3 kb Hind fragment in 2G6 was not cut by MboI indicates that the sites releasing the large fragments are in flanking DNA outside the marker sequences. Two enzymes sensitive to 5-methylcytosine were able to digest the marker DNA extensively, but the presence of small discreet fragments, such as that seen in 4F1, indicate that some cytosine residues may be methylated.

124-2D2#6: The major GUS-hybridizing sequences were present on two large fragments that separated from the bulk chromosomal DNA when electrophoresed without digestion (Figure 13A). The size of the smaller, ~7 kb

band was indistinguishable in size from the much stronger hybridization patterns obtained with either HindIII or EcoRI alone. These results indicate that the larger band is composed of repeats of the 7 kb hybridizing sequence that were released by HindIII and EcoRI digestion. These fragments were MboI resistant, but were apparently completely digested by either HpaII or MspI (not shown).

Small GUS-Hybridizing Fragments in 2G7#3 and 5A7#8: Figure 13B demonstrates that the GUS-hybridizing fragments are free from undigested chromosomal DNA, and that their size did not change when digested with either BamHI or EcoRI.

Analysis of Methylation in DNA Fragments Hybridizing to the Thaumatin Marker: Analysis of methylation in the 2.5 kb HindIII-EcoRI fragment hybridizing to a thaumatin probe in DNA from the F_3 plant, G12-2-8, and the F_2 plant, 12H2-4, was performed using MboI and MspI (Fig. 14). For both samples digested with a combination of the restriction enzymes, a 2.5 kb hybridizing fragment was produced. Following digestion with HindIII and EcoRI plus MspI, the 2.5 kb fragment was not digested completely. These results were substantially different from those obtained for the GUS-positive fragments and indicated that a substantial portion of the MboI sites in the target DNA were accessible to that enzyme, while the MspI sites were at least partly blocked.

More generally, these results showed that a transforming thaumatin gene that is stably inherited has the methylation pattern expected for most barley DNA; that is, there was little adenine methylation but some cytosine methylation. In contrast, the unstable JR133-series transforming genes, although apparently integrated

into chromosomal DNA, were marked by the presence of 6-methyladenine where the GATC sequence occurred and exhibited little methylation of the cytosine residues. As the unstable transforming genes were lost, intermediate forms that were free from the chromosomal DNA and displayed various deletions could be identified.

Transient expression was thus usefully effected by means of integrated DNA characterized by extensive adenine methylation, but heritably stable integration was accomplished when such methylation was minimized. This principle can be applied in selecting or designing other genes for stable expression according to the present invention.

EXAMPLE 6. TRANSFORMATION OF ANOTHER CEREAL (OATS)

Tillers from oat plants derived from seeds (obtained from Mangelsdorf Seed Company, St. Louis) were injected with DNA as described for barley plants in Example 2. At the stage where five leaves had developed and when the developing inflorescence could be palpated at about the level of the third leaf, oat tillers were injected with DNA from construct JR133. The seeds were then screened as described for barley in EXAMPLE 2. Figure 15 provides the results of the screen for seeds in plates 4 (only extract screen is shown), 5 (only media screen is shown) and 6. Figs. 15A and 15B demonstrate the results for the media screen and the extract screen, respectively. The results indicate that in both the media and extract, the seed in well 6A4 exhibited a strong positive fluorescent signal indicating the presence of the transforming DNA, while the other wells did not appear to produce a positive signal.

What is Claimed is:

1. A method for obtaining seed comprised of genetically transformed endosperm tissue, comprising the steps of

(A) providing a genetic construct comprised of (i) a regulatory element which is expressed at high levels in an endosperm cell; (ii) at least one DNA sequence that encodes a polypeptide, said DNA sequence being under the transcriptional control of said regulatory element; and (iii) a terminal-processing signal positioned downstream of said DNA sequence with regard to the direction of transcription,

(B) injecting said genetic construct into a floral tiller of a cereal plant prior to anthesis in said plant; and thereafter

(C) assaying seeds from the injected plant for the presence of an expression product of said DNA sequence in the endosperm of any of said seeds, thereby to identify a seed comprised of genetically transformed endosperm tissue.

2. A method according to Claim 1, wherein said DNA sequence has a (G+C) content that is greater than 50%.

3. A method according to Claim 1, wherein step (C) comprises assaying for said expression product by using an antibody that recognizes said expression product.

4. A method according to Claim 1, wherein said regulatory element is comprised of a promoter selected from the group consisting of the Amy32b promoter, the Amy6-4 promoter, and the Aleurain promoter.

5. A method according to Claim 1, wherein the cereal plant injected in step (B) is of a crop selected from the group consisting of wheat, barley, oats, sorghum, rye, millet, rice and maize.

6. A method according to Claim 1, wherein said DNA sequence encodes at least a portion of the thaumatin amino-acid sequence.

7. A method according to Claim 6, wherein said DNA sequence encodes a fusion product comprised of said portion of the thaumatin amino-acid sequence.

8. A method according to Claim 1, wherein step (C) comprises assaying said seeds for the presence of an expression product of said DNA sequence in an aleurone layer of any of said seeds.

9. A differentiated monocotyledonous plant that produces seeds comprised of endosperm tissue genetically transformed to express an exogenous DNA sequence which encodes a polypeptide.

10. A plant according to Claim 9, wherein said exogenous DNA sequence is a eukaryotic or prokaryotic structural sequence.

11. A plant according to Claim 10, wherein said exogenous DNA sequence has a (G+C) content of greater than 50%.

12. A plant according to Claim 9, wherein said exogenous DNA sequence (A) codes for a polypeptide also encoded by a naturally-occurring gene and (B) has a (G+C)

content that is higher than that of said naturally-occurring gene.

13. A plant according to Claim 9, wherein said exogenous DNA sequence encodes at least a portion of the thaumatin amino-acid sequence.

14. A plant according to Claim 13, wherein said exogenous DNA sequence encodes a fusion product comprised of said portion of the thaumatin amino-acid sequence.

15. A plant according to Claim 9, wherein said plant produces seeds that, when germinated, grow into plants that produce seeds comprised of endosperm tissue expressing said exogenous DNA sequence.

16. A plant according to Claim 9, wherein said seeds are comprised of aleurone tissue genetically transformed to express said exogenous DNA sequence.

17. A substantially uniform population of monocotyledonous plants that produce seeds comprised, respectively, of endosperm containing an exogenous protein.

18. A uniform population of plants according to Claim 17, wherein said plants are cereal plants.

19. A uniform population of plants according to Claim 17, wherein said plants produce seeds comprised, respectively, of aleurone tissue containing an exogenous protein.

20. A seed of a monocotyledonous plant, wherein said seed comprises endosperm comprised of a polypeptide expression product of exogenous DNA.

21. A process of producing a polypeptide, comprising the steps of

(A) producing genetically transformed endosperm tissue that expresses a genetic construct comprised of (i) a regulatory element which is expressed at high levels in an endosperm cell; (ii) at least one DNA sequence that encodes a polypeptide, said DNA sequence being under the transcriptional control of said regulatory element; and (iii) a terminal-processing signal positioned downstream of said DNA sequence with respect to the direction of transcription, and

(B) isolating said polypeptide.

22. A process according to Claim 21, wherein said transformed endosperm tissue is transformed aleurone tissue.

23. A process according to Claim 21, wherein step (A) comprises producing a substantially uniform population of cereal plants that produce seeds comprised, respectively, of endosperm containing an exogenous protein; and step (B) comprises isolating said exogenous protein from said seeds.

24. A process according to Claim 21, wherein step (A) comprises maintaining, in a culture medium, endosperm tissue that is genetically transformed to express an exogenous DNA sequence which encodes a polypeptide; and step (B) comprises isolating said polypeptide from said culture medium.

FIG. 1A

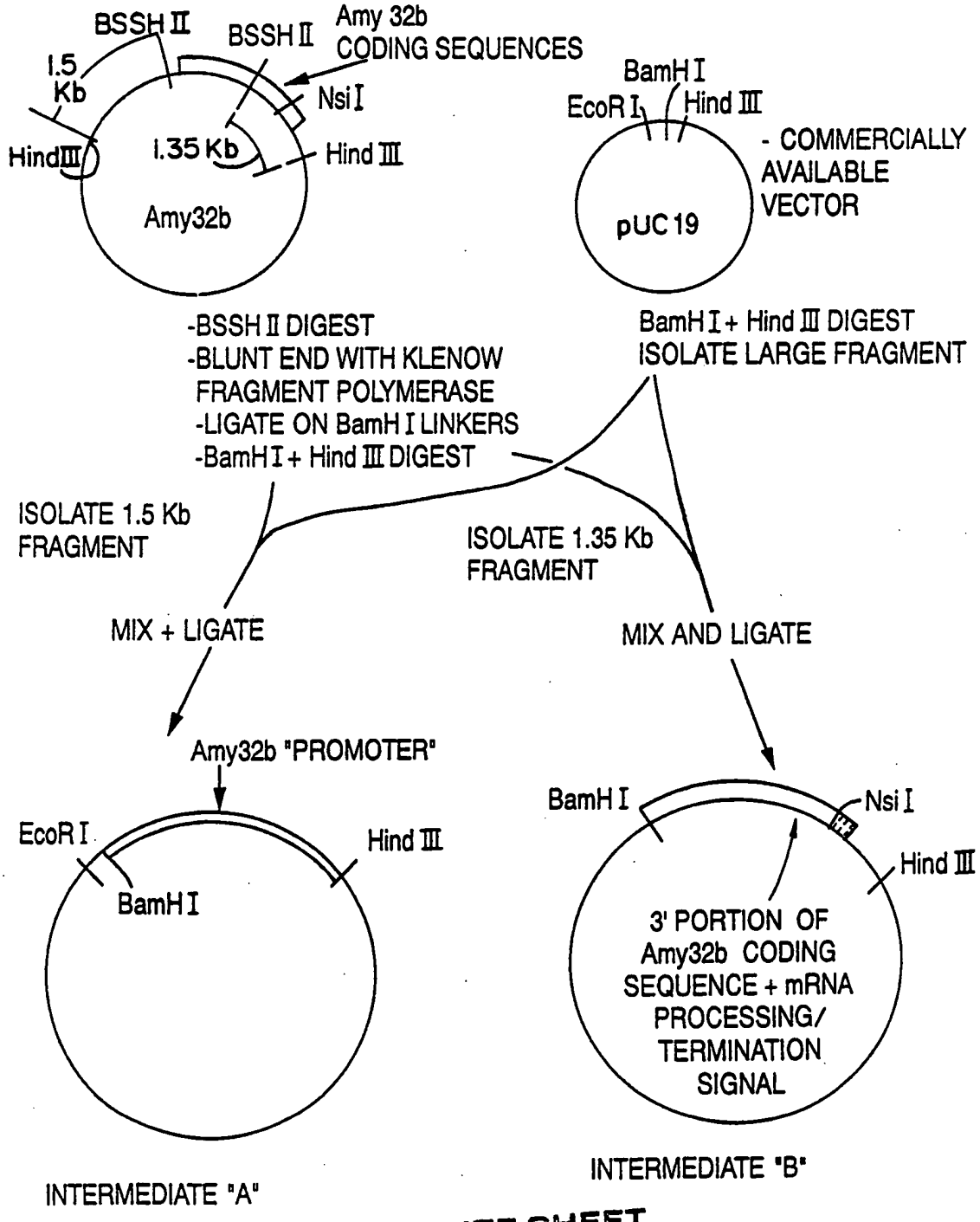

BSSH II
BSSH II
Amy 32b
CODING SEQUENCES
1.5
Kb
Nsi I
HindIII
1.35 Kb
Hind III
Amy32b
BamH I
EcoR I
Hind III
- COMMERCIALLY
AVAILABLE
VECTOR
pUC 19
-BSSH II DIGEST
-BLUNT END WITH KLENOW
FRAGMENT POLYMERASE
-LIGATE ON BamH I LINKERS
-BamH I + Hind III DIGEST
BamH I + Hind III DIGEST
ISOLATE LARGE FRAGMENT
ISOLATE 1.5 Kb
FRAGMENT
ISOLATE 1.35 Kb
FRAGMENT
MIX + LIGATE
MIX AND LIGATE
Amy32b "PROMOTER"
EcoR I
Hind III
BamH I
BamH I
Nsi I
Hind III
3' PORTION OF
Amy32b CODING
SEQUENCE + mRNA
PROCESSING/
TERMINATION
SIGNAL
INTERMEDIATE "A"
INTERMEDIATE "B"

FIG. 1B

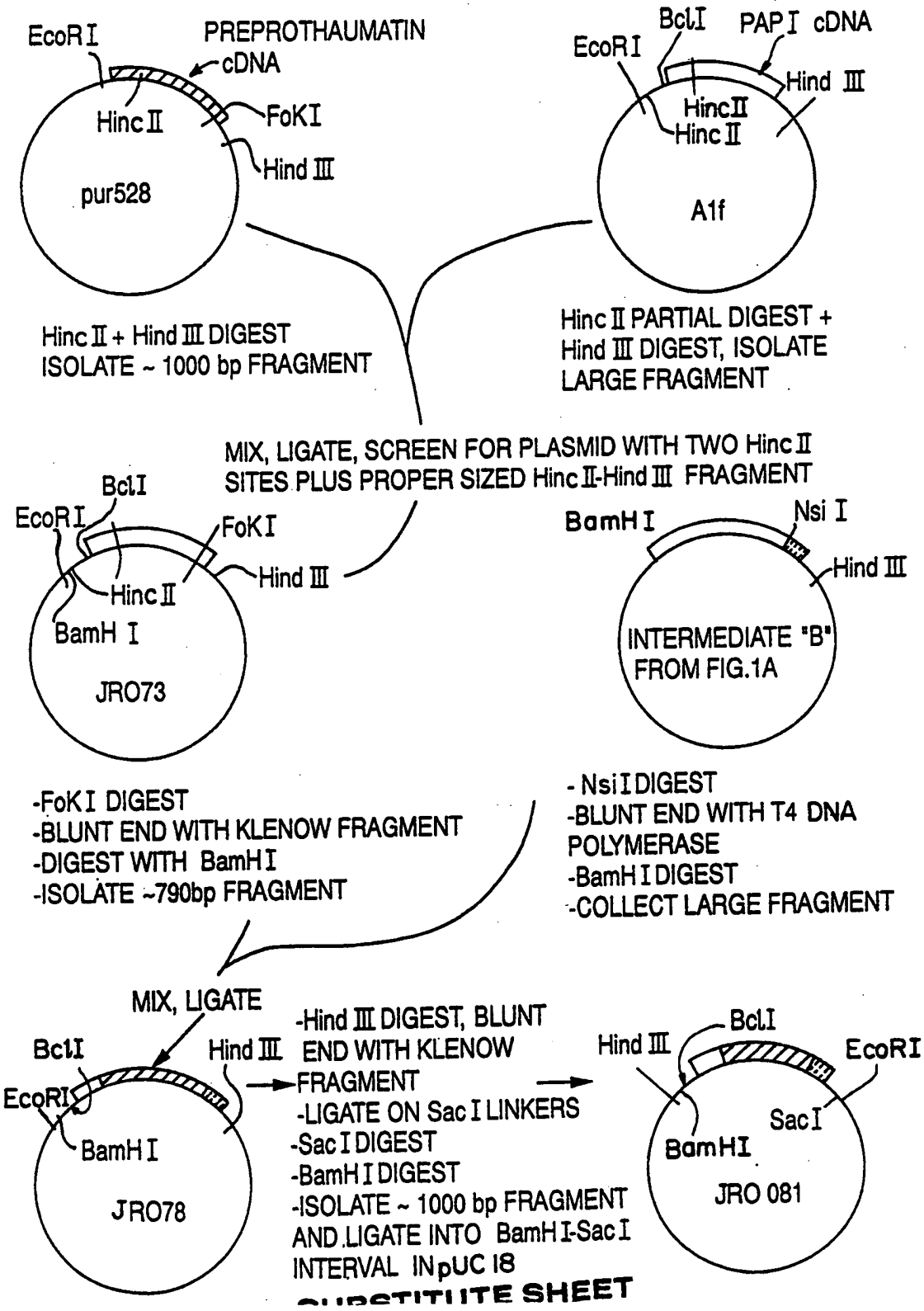


FIG. 1C

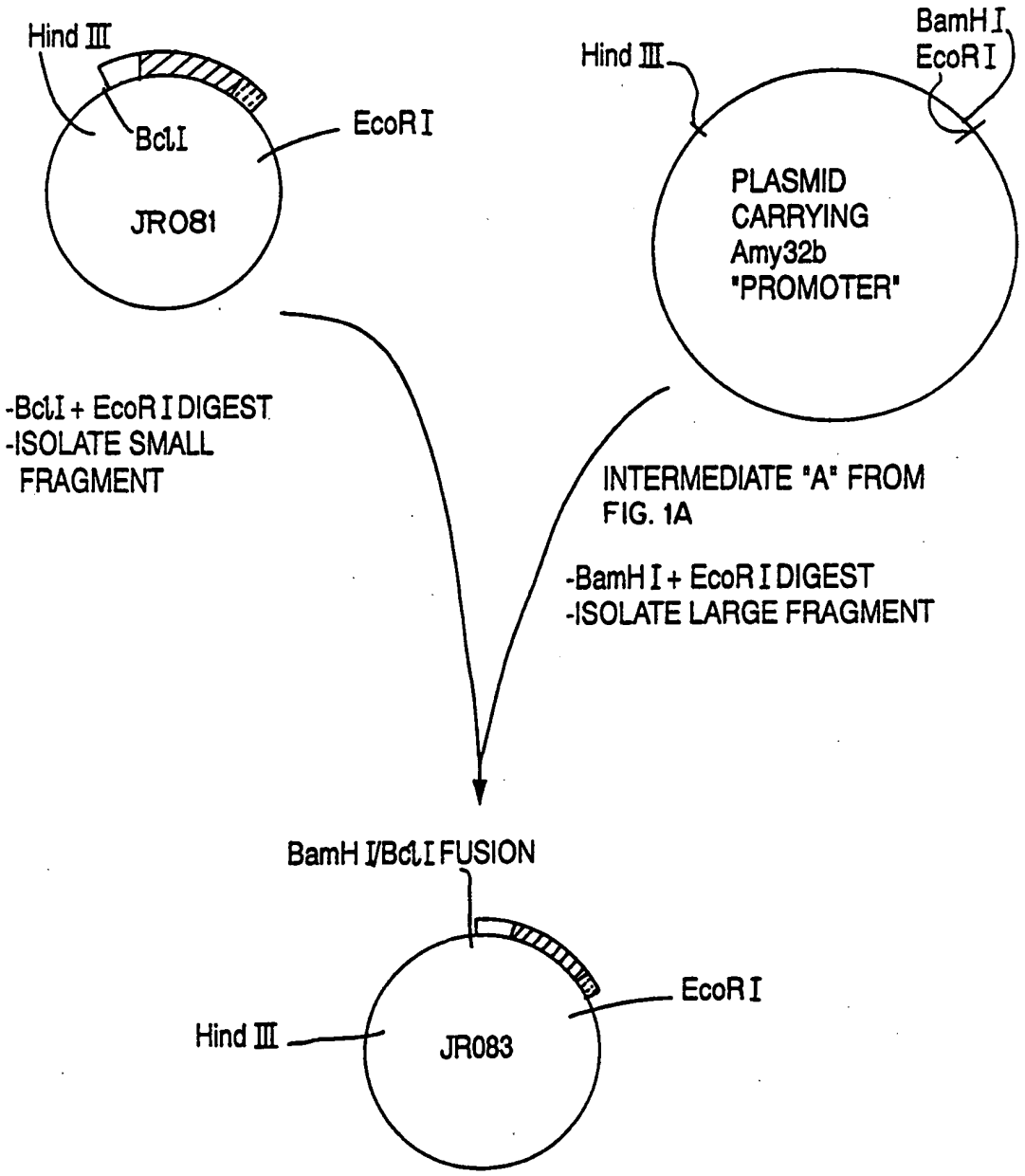

Hind III
BclI
EcoR I
JR081
-BclI + EcoR I DIGEST
-ISOLATE SMALL FRAGMENT
Hind III
BamH I
EcoR I
PLASMID CARRYING Amy32b "PROMOTER"
INTERMEDIATE "A" FROM FIG. 1A
-BamH I + EcoR I DIGEST
-ISOLATE LARGE FRAGMENT
BamH I/BclI FUSION
Hind III
JR083
EcoR I

FIG. 2

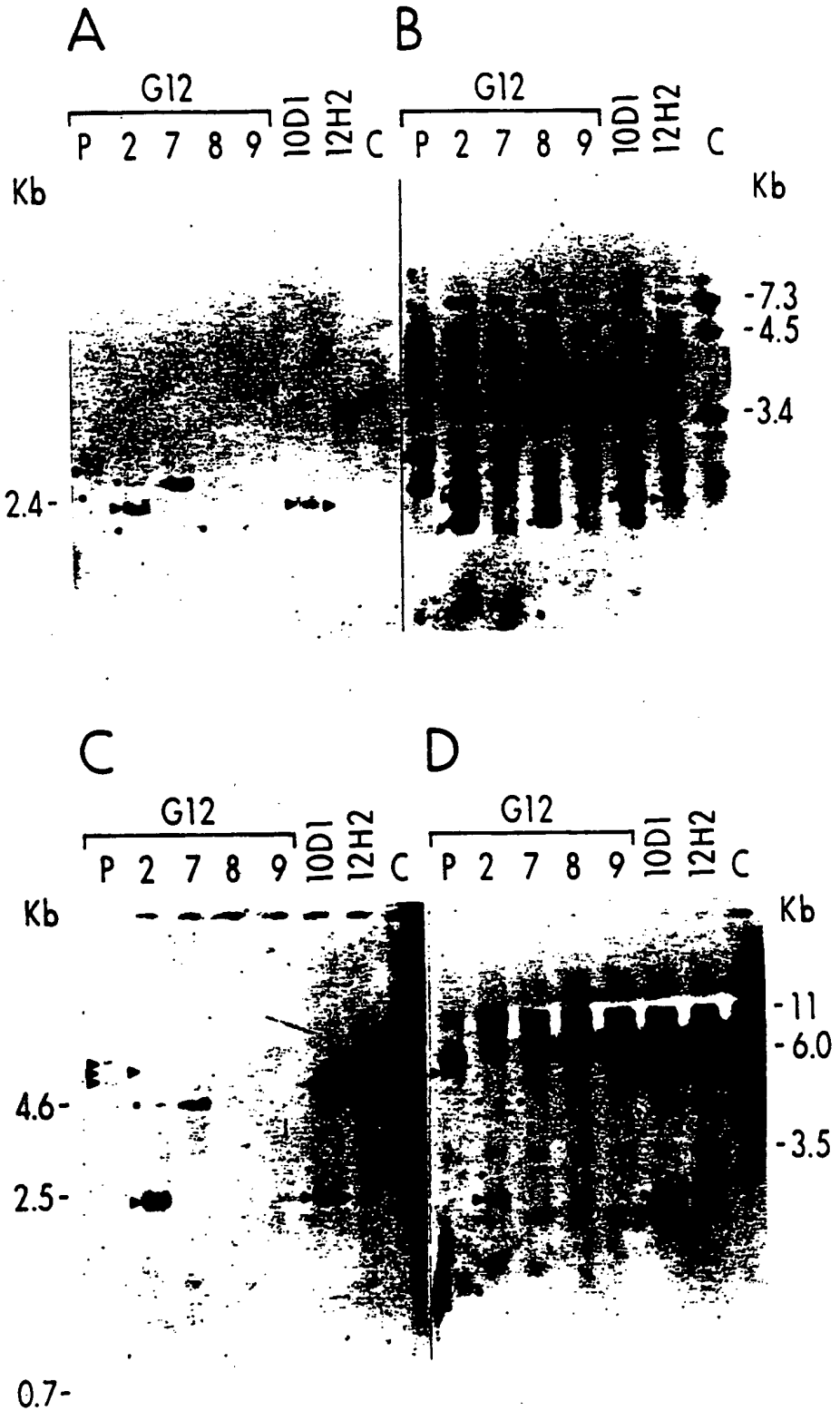


FIG. 3

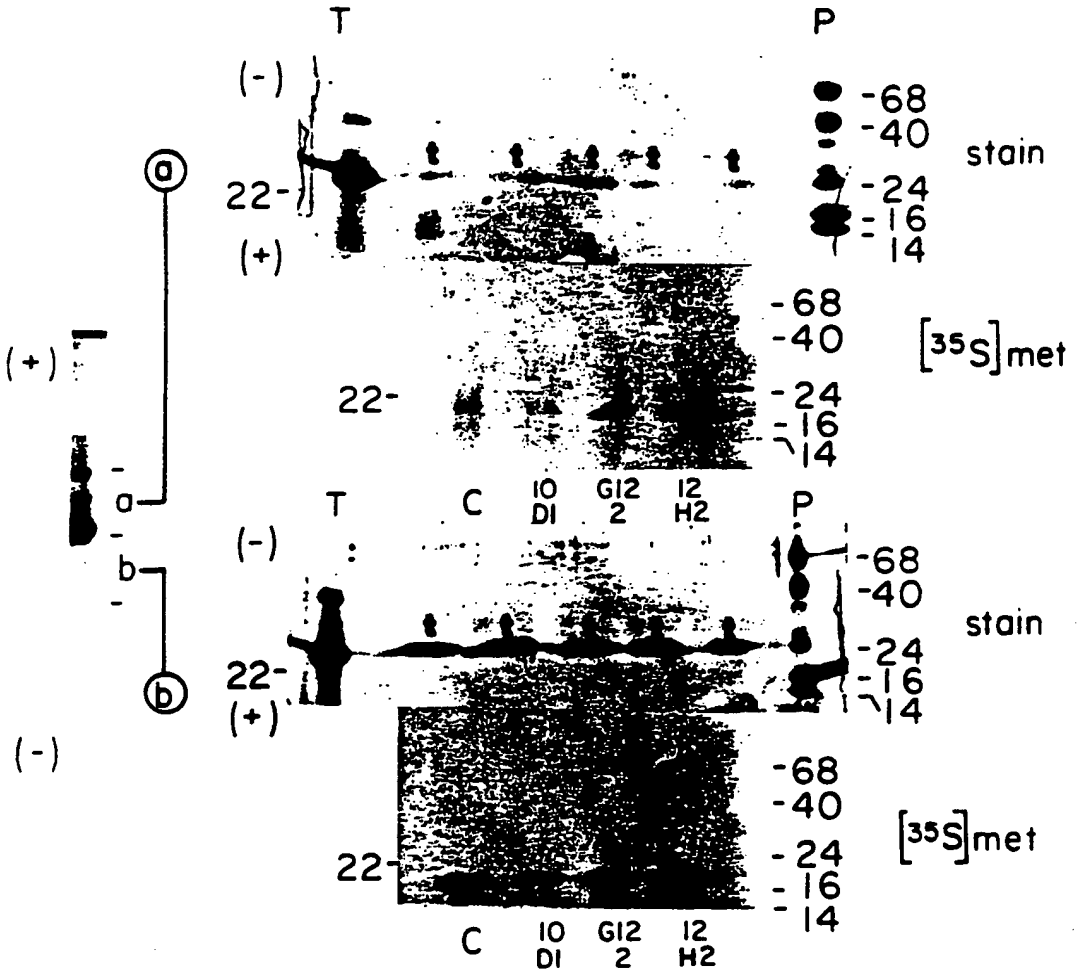


FIG. 4A

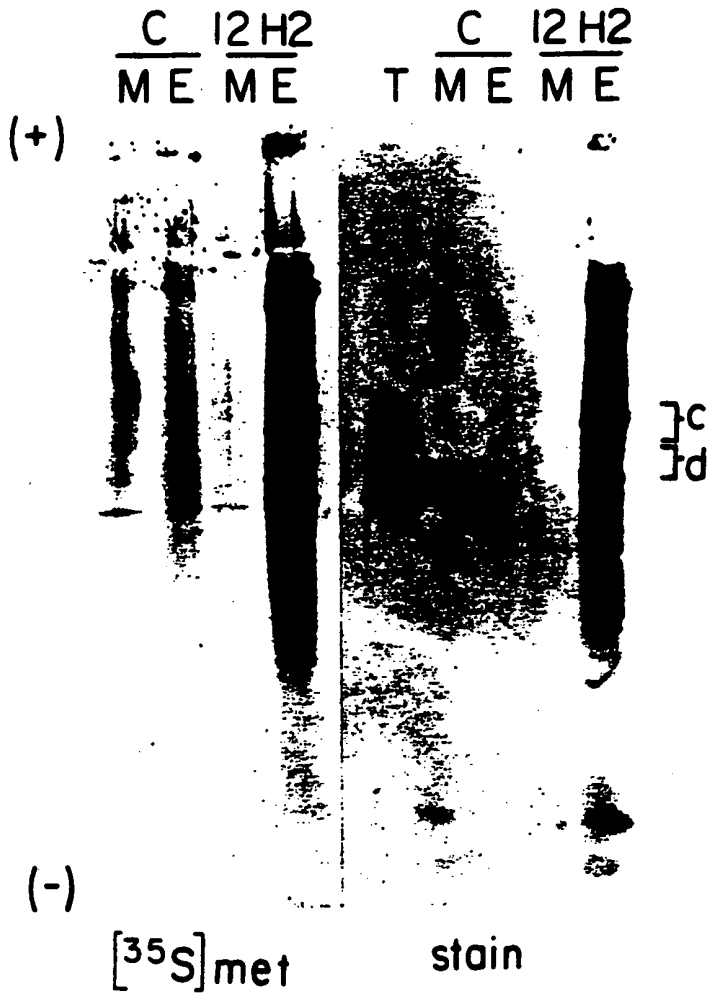


FIG. 4B

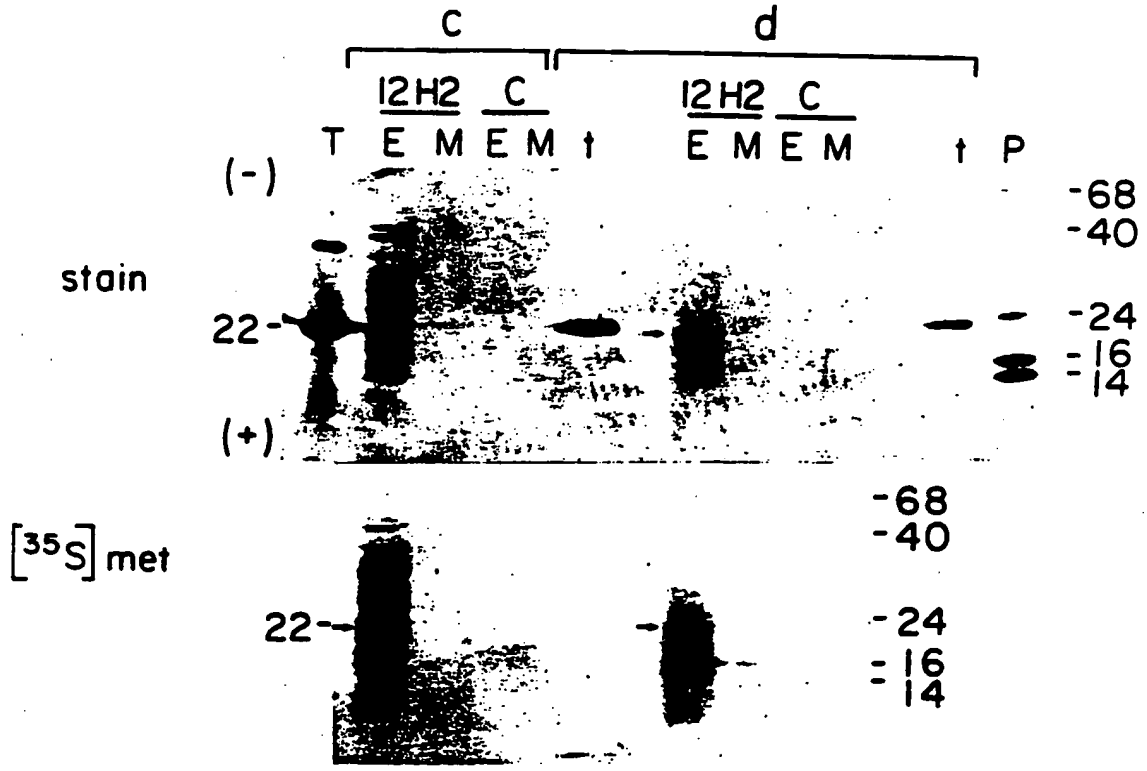

c
d
12H2 C
12H2 C
T E M E M t E M E M t P
(-)
stain
22
(+)
-68
-40
-24
-16
-14
[35S] met
22
-68
-40
-24
-16
-14

FIG. 5A(1)

CONSTRUCTION OF JR124,WHERE THE CODING SEQUENCE OF E. Col:
β-GLUCURONIDASE (GUS) IS BRACKETED BY THE Amy32b PROMOTER/ UPSTREAM SEQUENCES AND THE Amy32b 3' SEQUENCES FOR RNA PROCESSING /POLY(A) ADDITION:

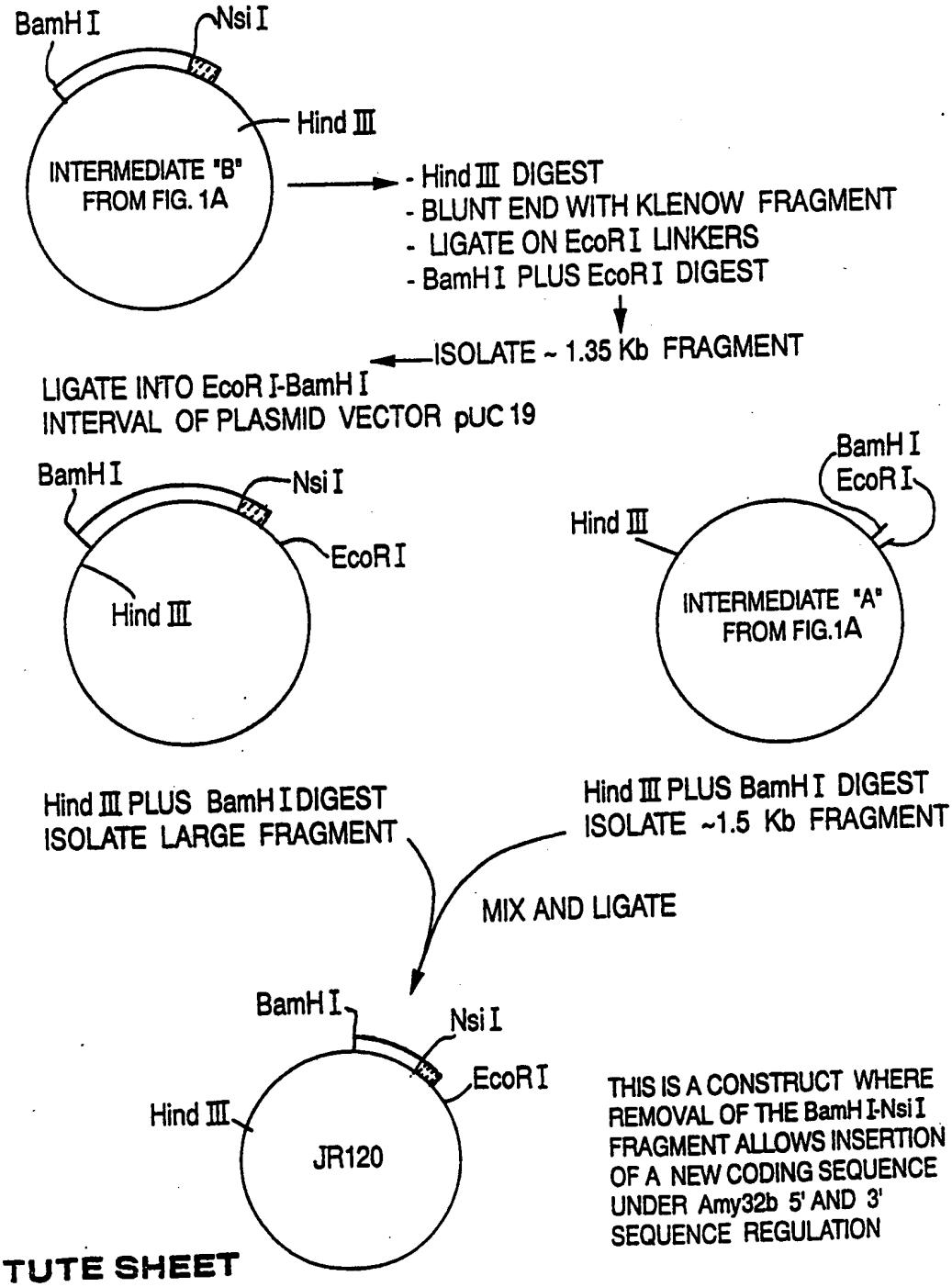


FIG. 5A(2)

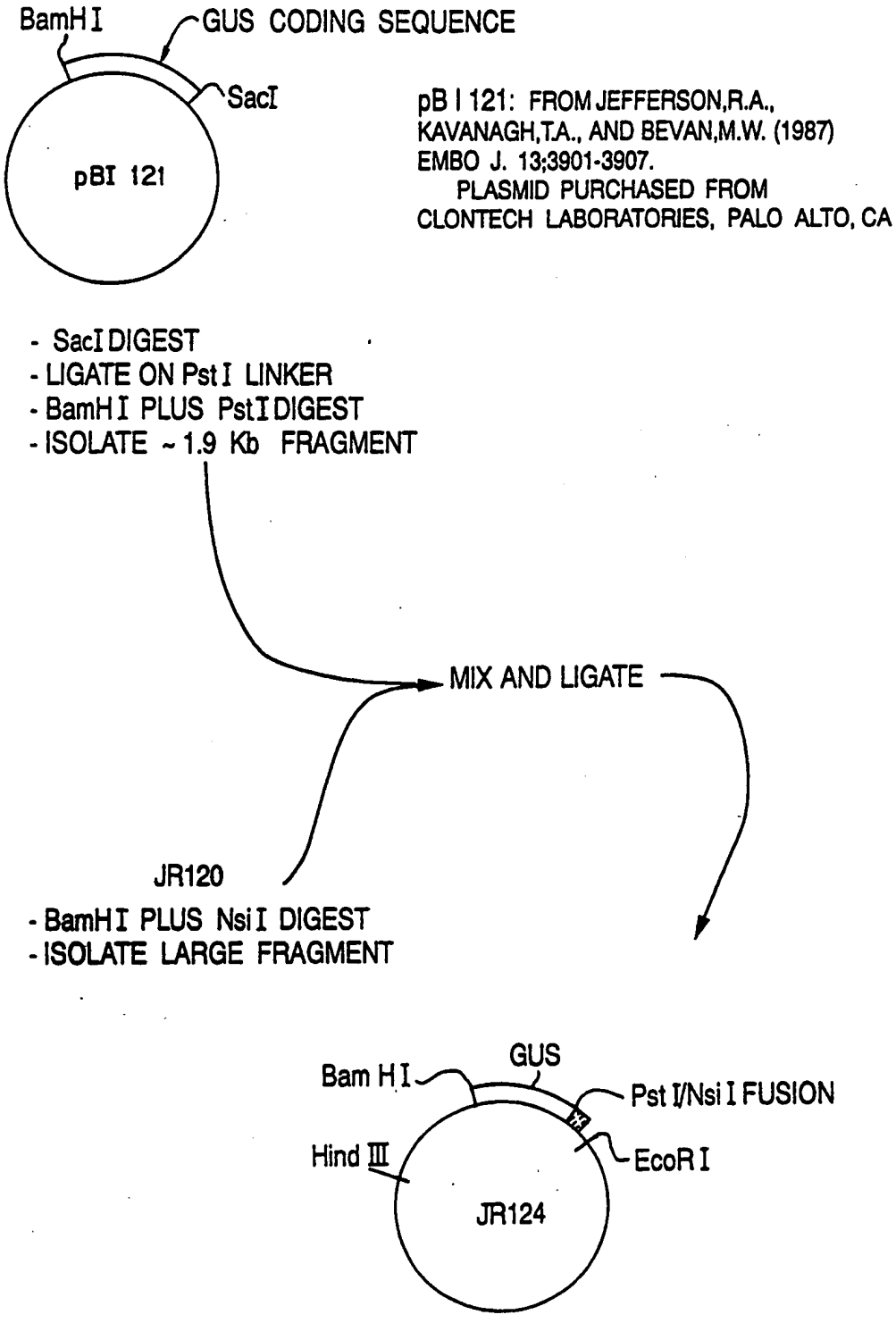

BamHI
GUS CODING SEQUENCE
SacI
pBI 121
pBI 121: FROM JEFFERSON,R.A., KAVANAGH,T.A., AND BEVAN,M.W. (1987) EMBO J. 13;3901-3907.
PLASMID PURCHASED FROM CLONTECH LABORATORIES, PALO ALTO, CA
- SacI DIGEST
- LIGATE ON PstI LINKER
- BamHI PLUS PstI DIGEST
- ISOLATE ~1.9 Kb FRAGMENT
MIX AND LIGATE
JR120
- BamHI PLUS NsiI DIGEST
- ISOLATE LARGE FRAGMENT
Bam HI
GUS
Pst I/Nsi I FUSION
Hind III
EcoR I
JR124

FIG. 5B(1)

CONSTRUCTION OF JR129, WHERE THE PROMOTER/UPSTREAM SEQUENCES AND PART OF THE CODING SEQUENCE FOR THE BARLEY GENE ALEURAIN (WHITTIER,R.F., DEAN, D. A., AND ROGERS, J. C. (1987) NUCLEIC ACIDS RES. 15,2515 - 2535) ARE FUSED TO GUS:

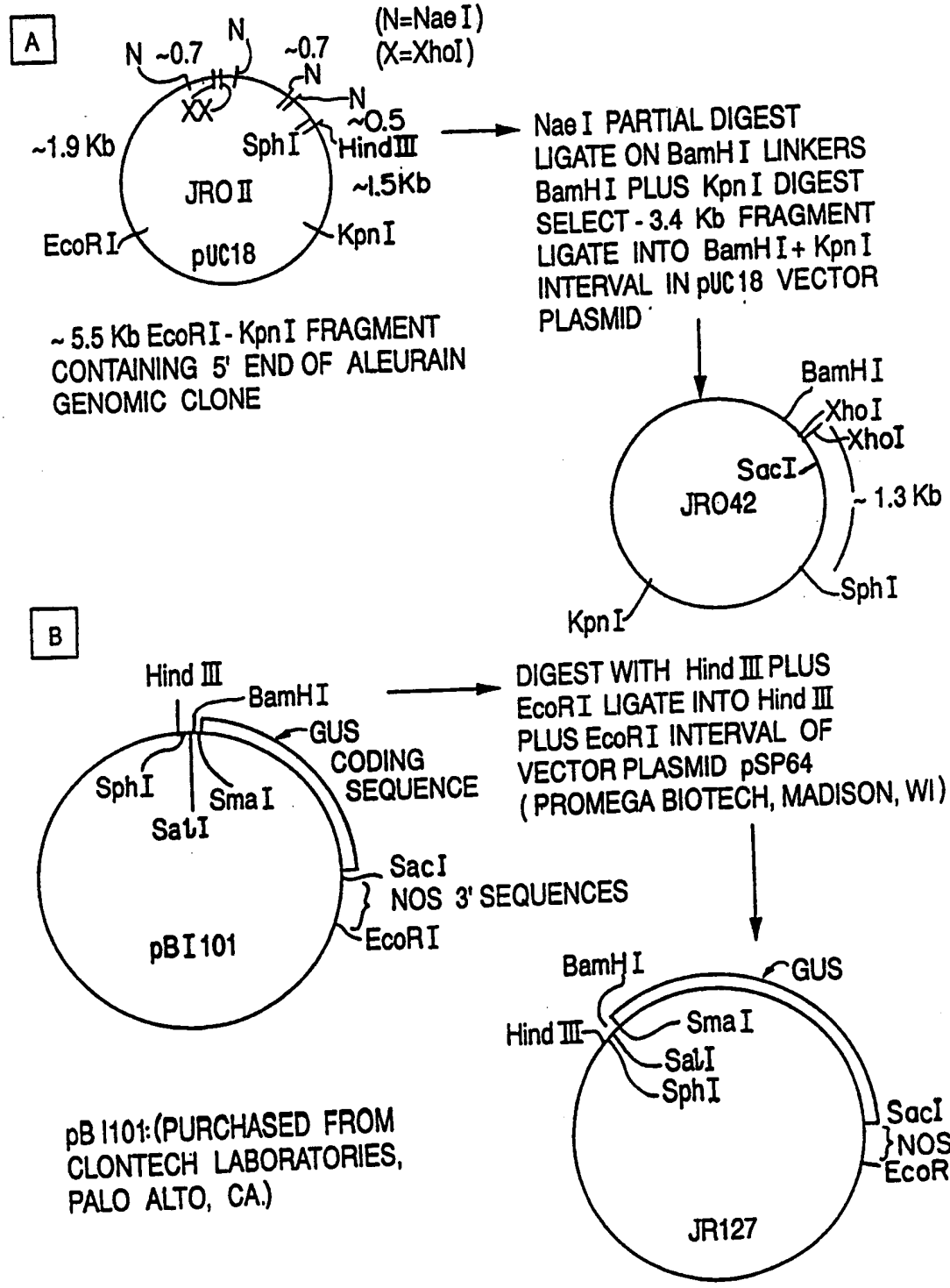


FIG. 5B(2)

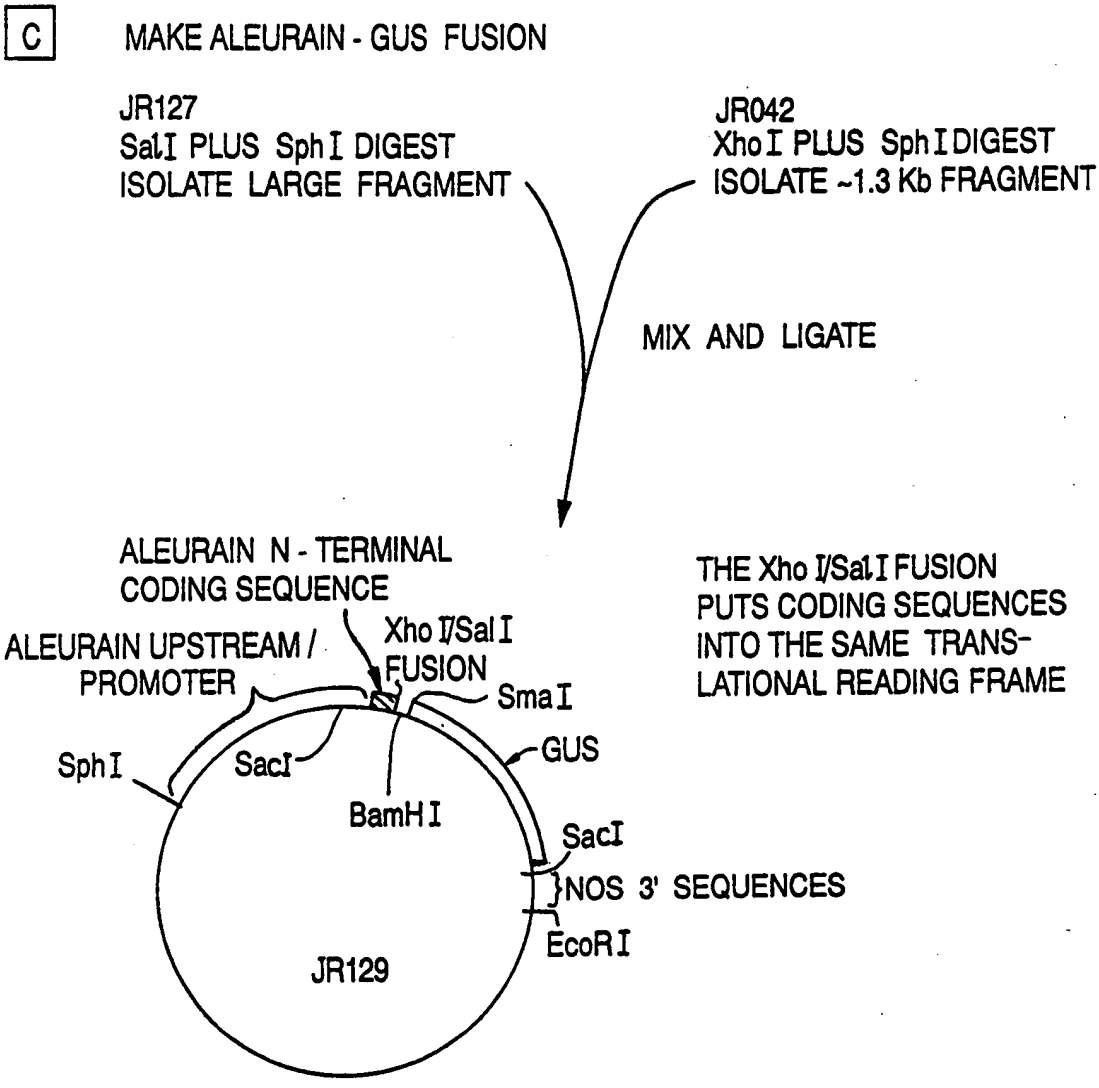

C
MAKE ALEURAIN - GUS FUSION
JR127
SalI PLUS SphI DIGEST
ISOLATE LARGE FRAGMENT
JR042
XhoI PLUS SphI DIGEST
ISOLATE ~1.3 Kb FRAGMENT
MIX AND LIGATE
ALEURAIN N - TERMINAL
CODING SEQUENCE
ALEURAIN UPSTREAM /
PROMOTER
XhoI/SalI
FUSION
SmaI
SphI
SacI
GUS
BamHI
SacI
NOS 3' SEQUENCES
EcoRI
JR129
THE XhoI/SalI FUSION
PUTS CODING SEQUENCES
INTO THE SAME TRANS-
LATIONAL READING FRAME

FIG. 5C(1)

CONSTRUCTION OF JR133,WHERE THE GUS CODING SEQUENCE IS FUSED TP THE N - TERMINAL CODING SEQUENCE OF THE BARLEY α- AMYLASE GENE ,Amy6-4 (KLURSHEED,B.,AND ROGERS,J. C. (1988) J. BIOL. CHEM. 263, 18953 - 18960). THE COMPLETED CONSTRUCT BRACKETS THE FUSED CODING SEQUENCE BETWEEN THE Amy6-4 UPSTREAM / PROMOTER REGION AND THEAmy6-4 3' SEQUENCES FOR RNA PROCESSING / POLY (A) ADDITION.

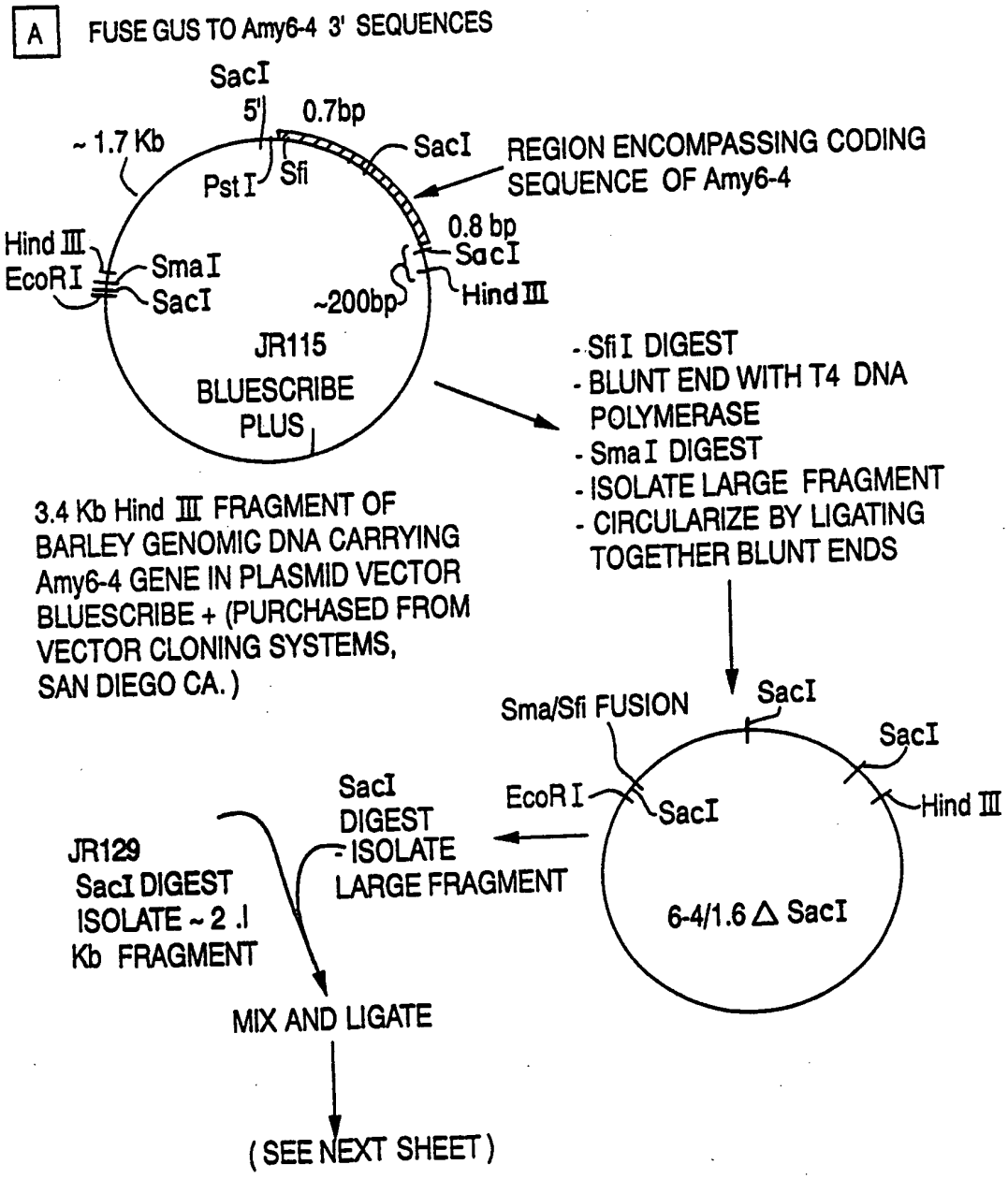


FIG. 5C(2)

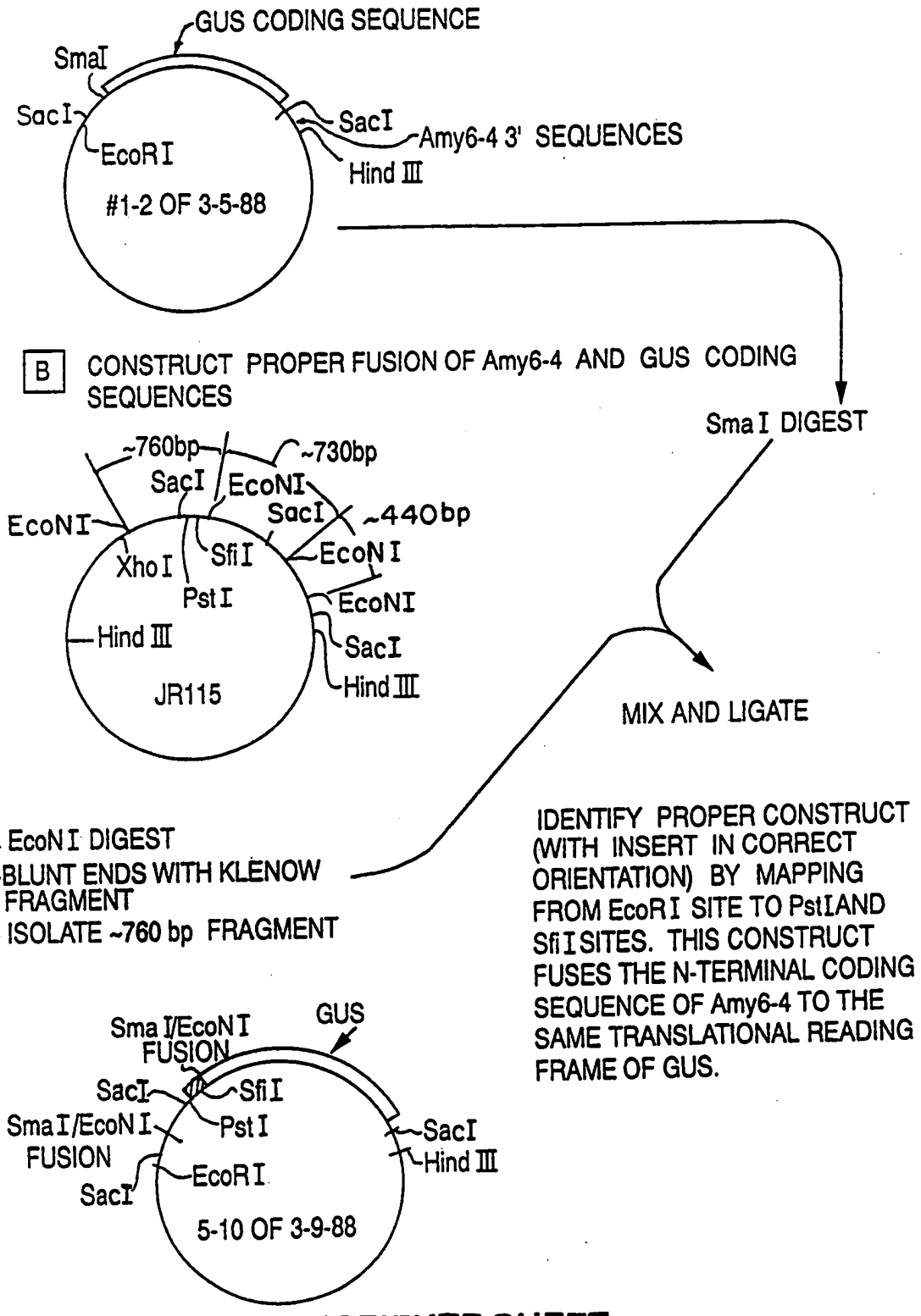

GUS CODING SEQUENCE
SmaI
SacI
EcoRI
#1-2 OF 3-5-88
SacI
Amy6-4 3' SEQUENCES
Hind III
B
CONSTRUCT PROPER FUSION OF Amy6-4 AND GUS CODING SEQUENCES
Sma I DIGEST
~760bp
~730bp
SacI
EcoNI
SacI
~440bp
EcoNI
XhoI
SfiI
PstI
EcoNI
EcoNI
Hind III
SacI
JR115
Hind III
MIX AND LIGATE
- EcoN I DIGEST
-BLUNT ENDS WITH KLENOW FRAGMENT
- ISOLATE ~760 bp FRAGMENT
IDENTIFY PROPER CONSTRUCT (WITH INSERT IN CORRECT ORIENTATION) BY MAPPING FROM EcoR I SITE TO PstI AND SfiI SITES. THIS CONSTRUCT FUSES THE N-TERMINAL CODING SEQUENCE OF Amy6-4 TO THE SAME TRANSLATIONAL READING FRAME OF GUS.
Sma I/EcoN I FUSION
GUS
SacI
SfiI
Sma I/EcoN I FUSION
PstI
SacI
Hind III
SacI
EcoRI
5-10 OF 3-9-88

FIG. 5C(3)

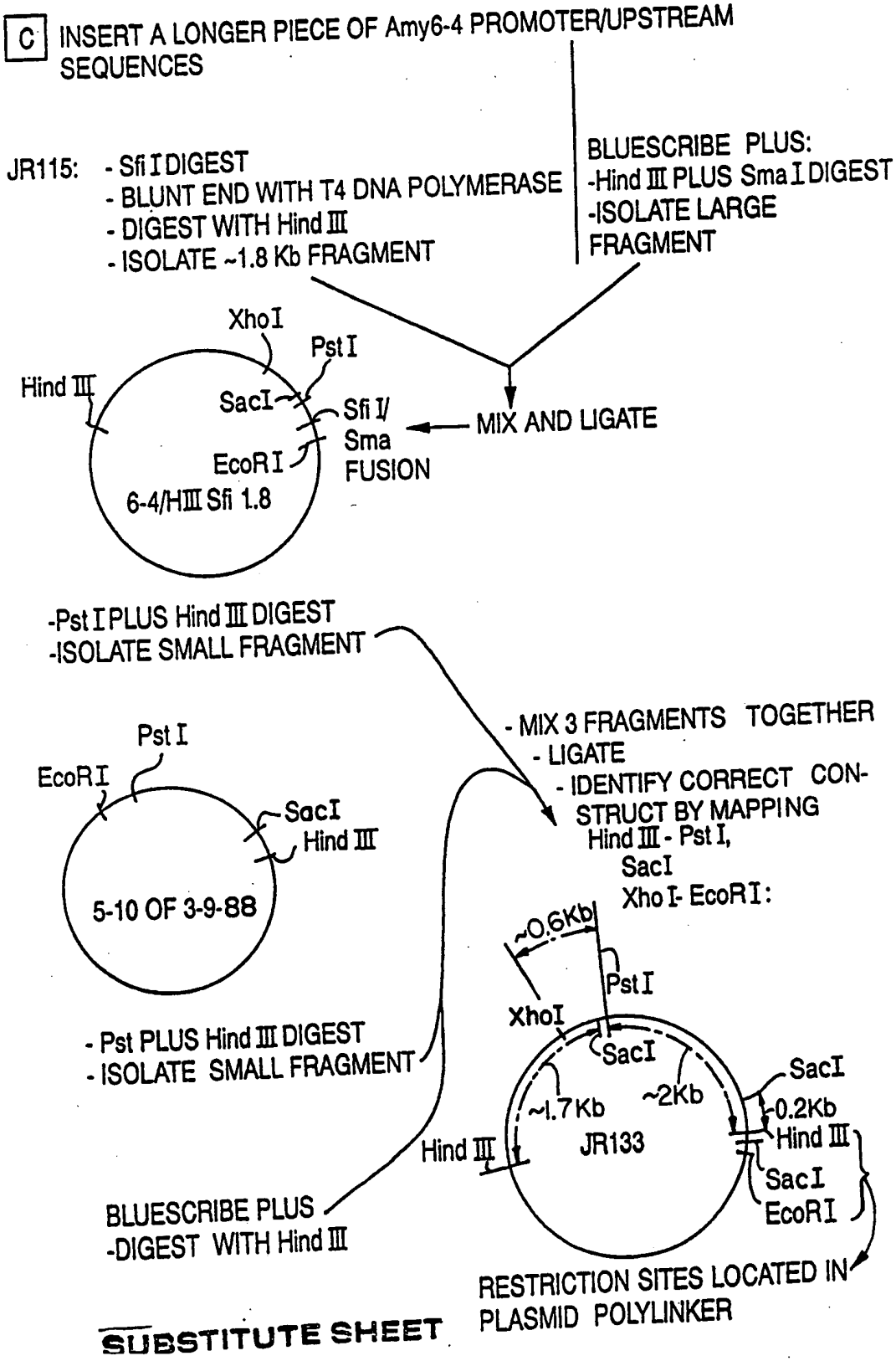

C INSERT A LONGER PIECE OF Amy6-4 PROMOTER/UPSTREAM SEQUENCES
JR115: - Sfi I DIGEST
- BLUNT END WITH T4 DNA POLYMERASE
- DIGEST WITH Hind III
- ISOLATE ~1.8 Kb FRAGMENT
BLUESCRIBE PLUS:
-Hind III PLUS Sma I DIGEST
-ISOLATE LARGE FRAGMENT
XhoI
PstI
Hind III
SacI
Sfi I/ Sma FUSION
MIX AND LIGATE
EcoRI
6-4/HIII Sfi 1.8
-Pst I PLUS Hind III DIGEST
-ISOLATE SMALL FRAGMENT
- MIX 3 FRAGMENTS TOGETHER
- LIGATE
- IDENTIFY CORRECT CONSTRUCT BY MAPPING
Hind III - Pst I,
SacI
Xho I - EcoRI:
Pst I
EcoRI
SacI
Hind III
5-10 OF 3-9-88
~0.6Kb
PstI
XhoI
SacI
- Pst PLUS Hind III DIGEST
- ISOLATE SMALL FRAGMENT
~1.7Kb
~2Kb
SacI
0.2Kb
Hind III
JR133
Hind III
SacI
EcoRI
BLUESCRIBE PLUS
-DIGEST WITH Hind III
RESTRICTION SITES LOCATED IN PLASMID POLYLINKER

FIG. 6A

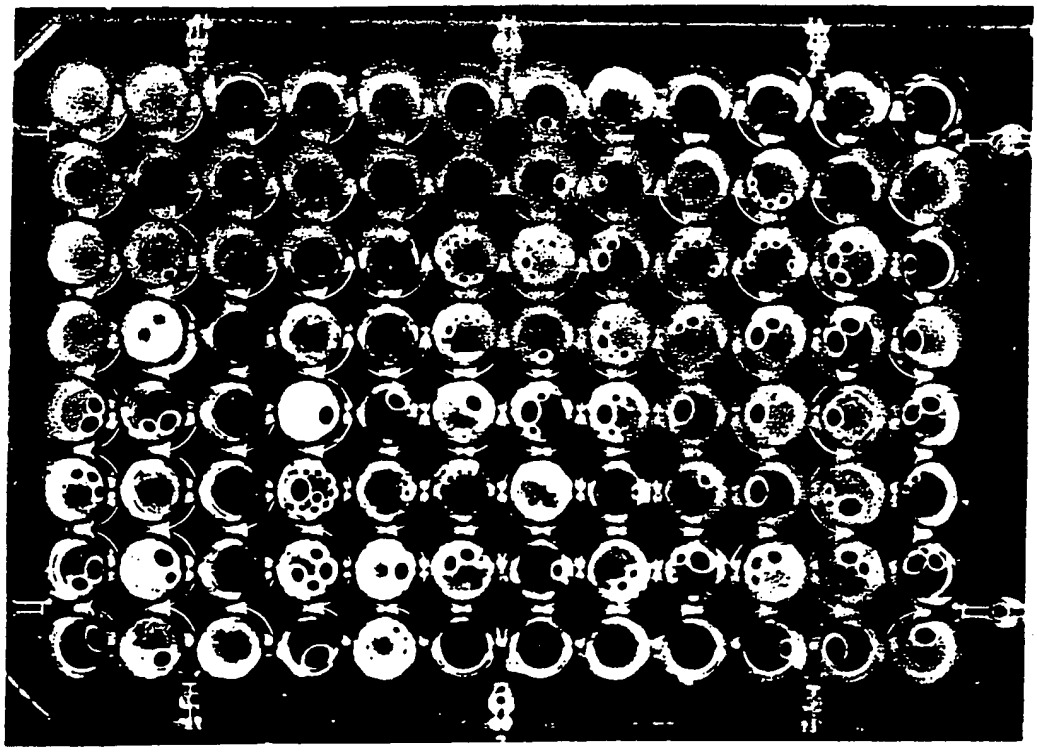


FIG. 6B

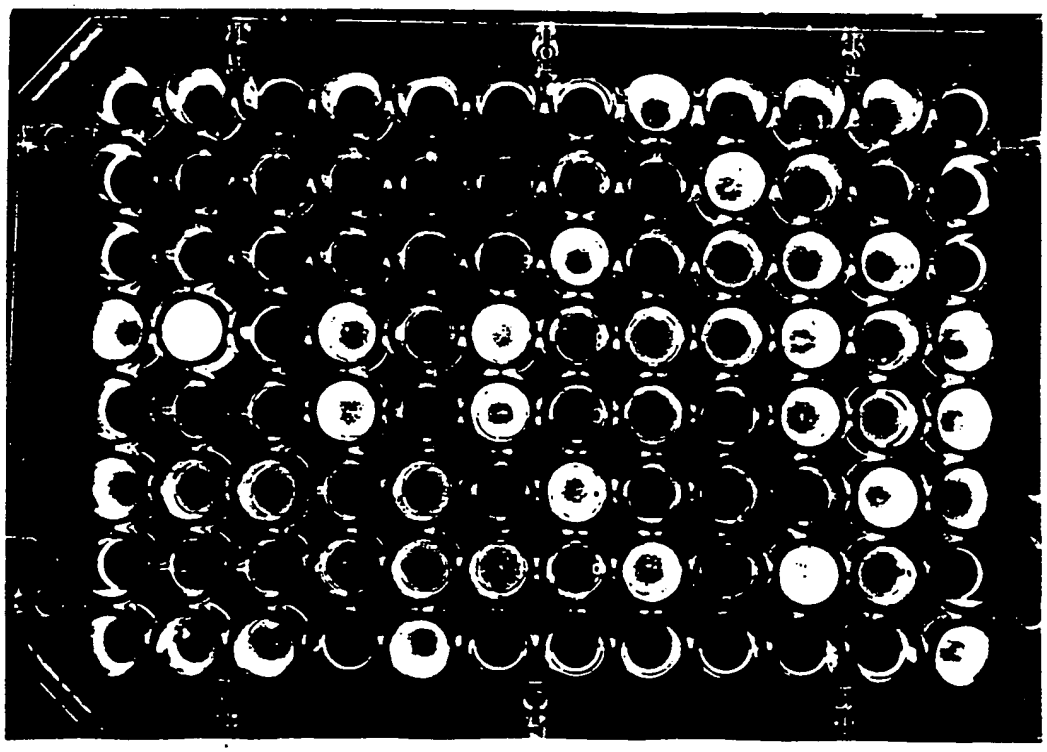


FIG. 7

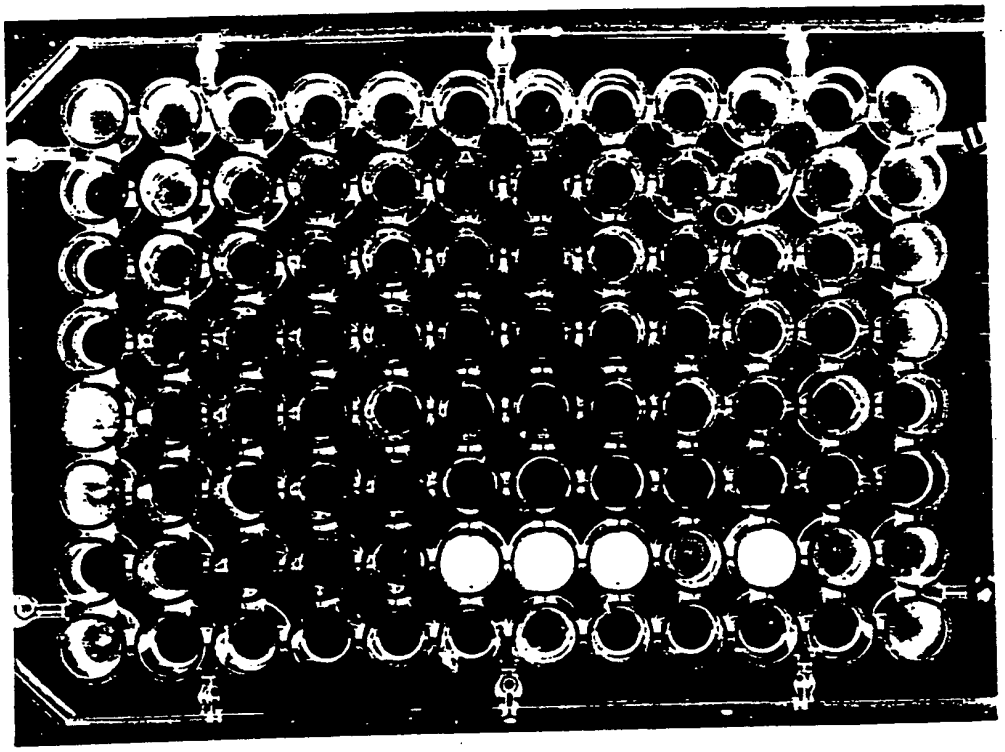


FIG. 8

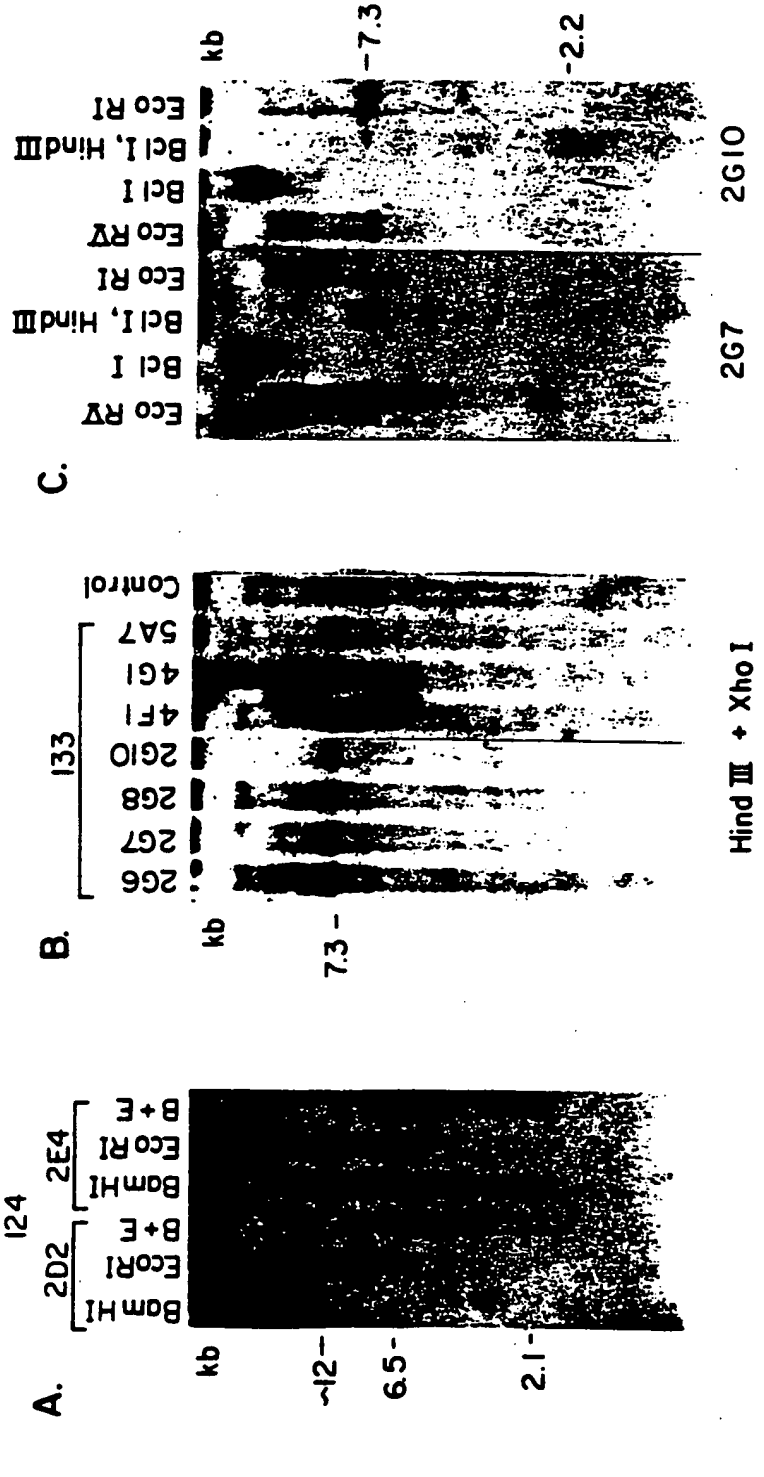

A.
kb
~12
6.5
2.1
124
2D2
BamHI
EcoRI
B+E
2E4
BamHI
EcoRI
B+E
B.
kb
7.3
133
2G6
2G7
2G8
2G10
4F1
4G1
5A7
Control
Hind III + Xho I
C.
kb
7.3
2.2
Eco RI
Bcl I, HindIII
Bcl I
Eco RV
Eco RI
Bcl I, HindIII
Bcl I
Eco RV
2G7
2G10

FIG. 10

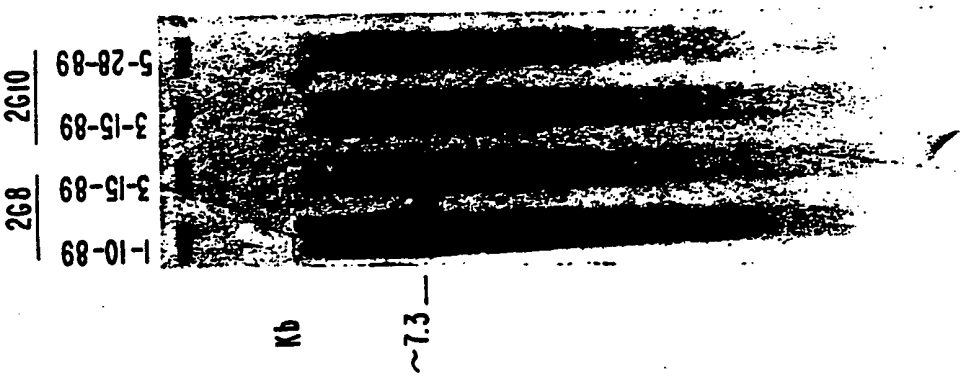

2G8
1-10-89
3-15-89
2G10
3-15-89
5-28-89
Kb
~7.3

FIG. 9

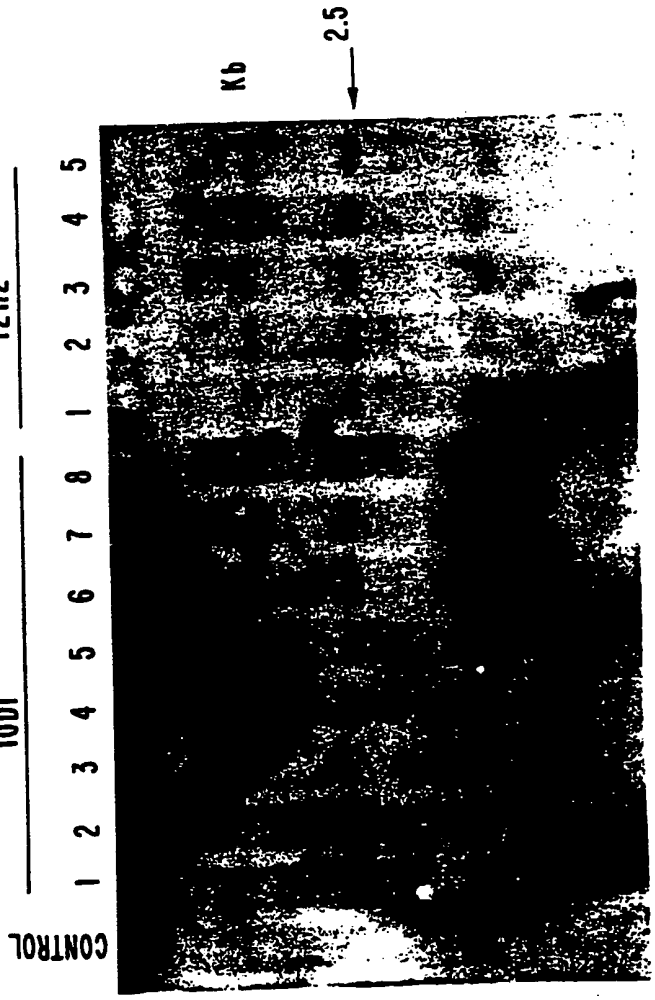

Kb
2.5
CONTROL
1 2 3 4 5 6 7 8
10D1
1 2 3 4 5
12H2

FIG. 11

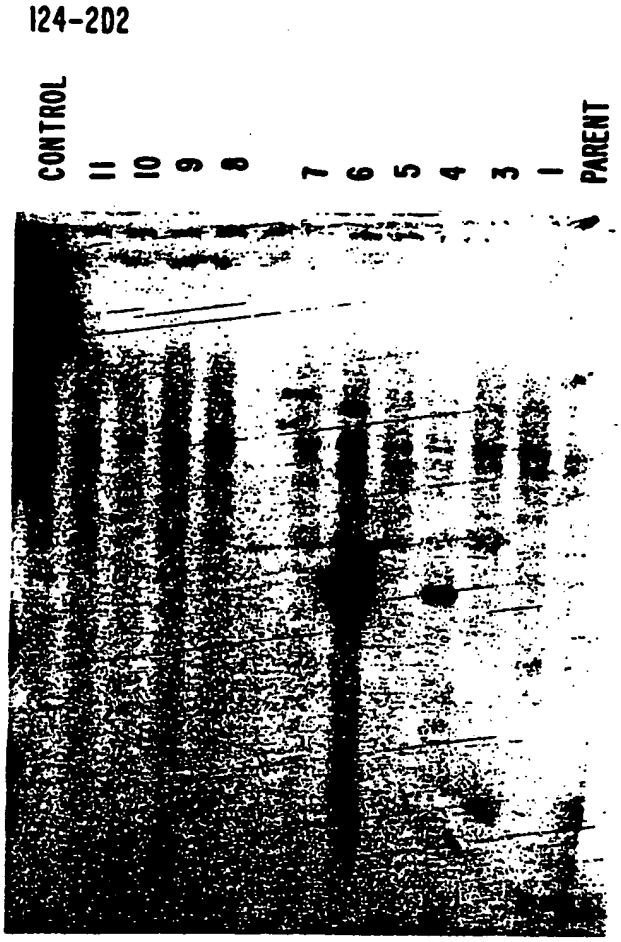


FIG. 12

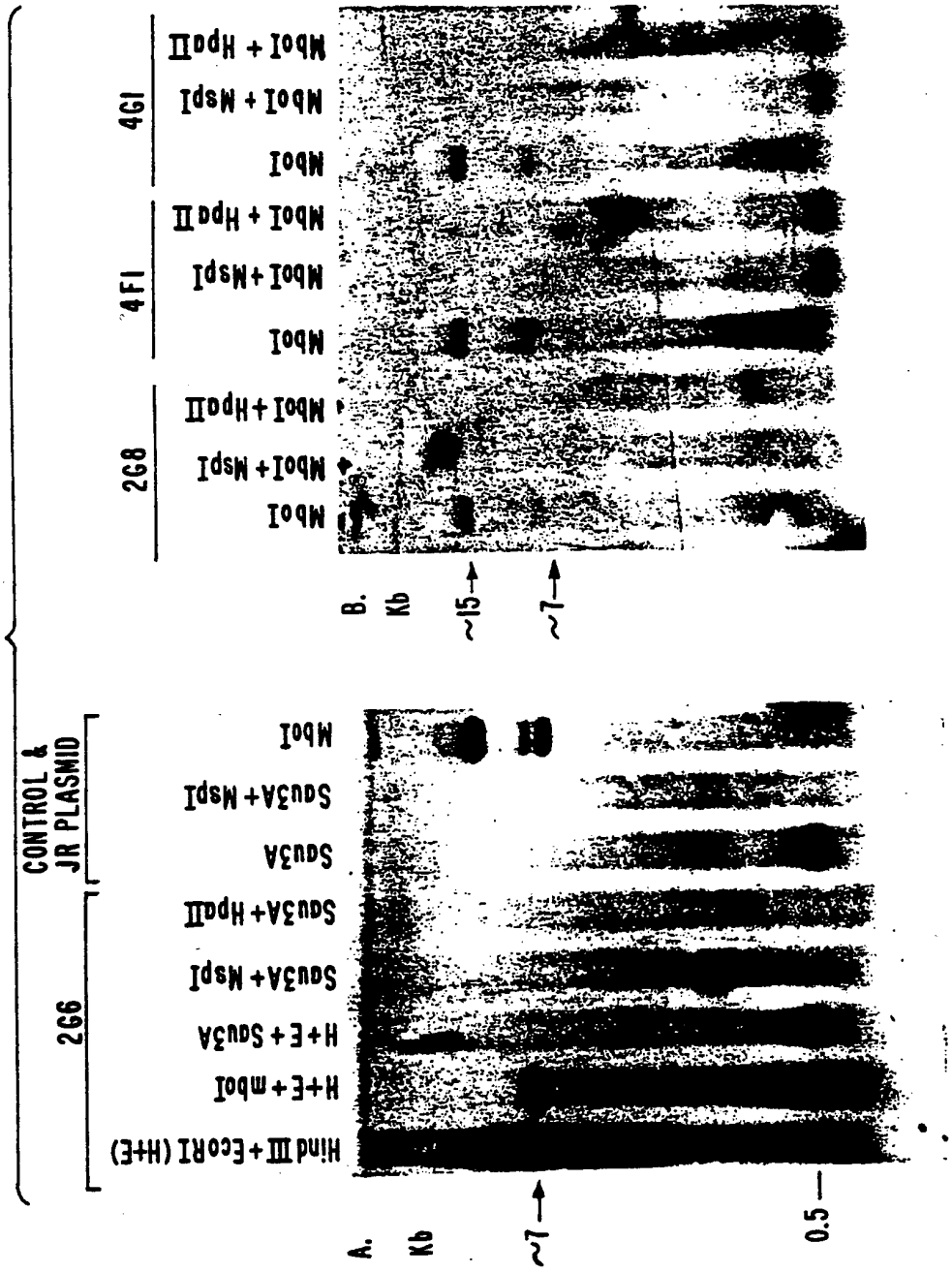

MboI + HpaII
MboI + MspI
MboI
MboI + HpaII
MboI + MspI
MboI
MboI + HpaII
MboI + MspI
MboI
4G1
4F1
2G8
B.
Kb
~15
~7
MboI
Sau3A + MspI
Sau3A
Sau3A + HpaII
Sau3A + MspI
H + E + Sau3A
H + E + mboI
HindIII + EcoRI (H+E)
CONTROL & JR PLASMID
2G6
A.
Kb
~7
0.5

FIG. 13

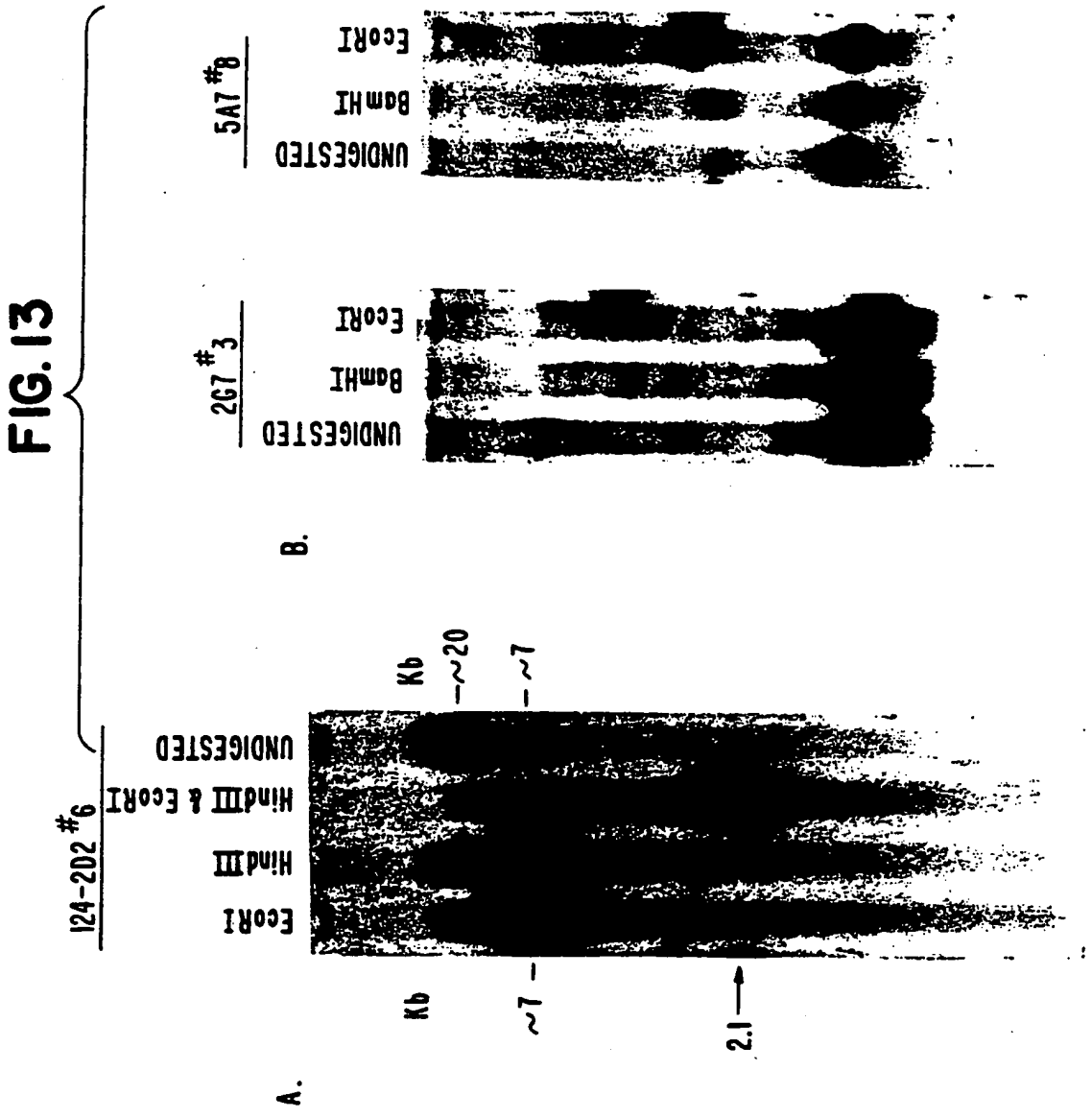

B.
5A7 #8
EcoRI
BamHI
UNDIGESTED
2G7 #3
EcoRI
BamHI
UNDIGESTED
A.
124-202 #6
EcoRI
HindIII
HindIII & EcoRI
UNDIGESTED
Kb
~20
~7
~7
Kb
2.1

FIG. 14

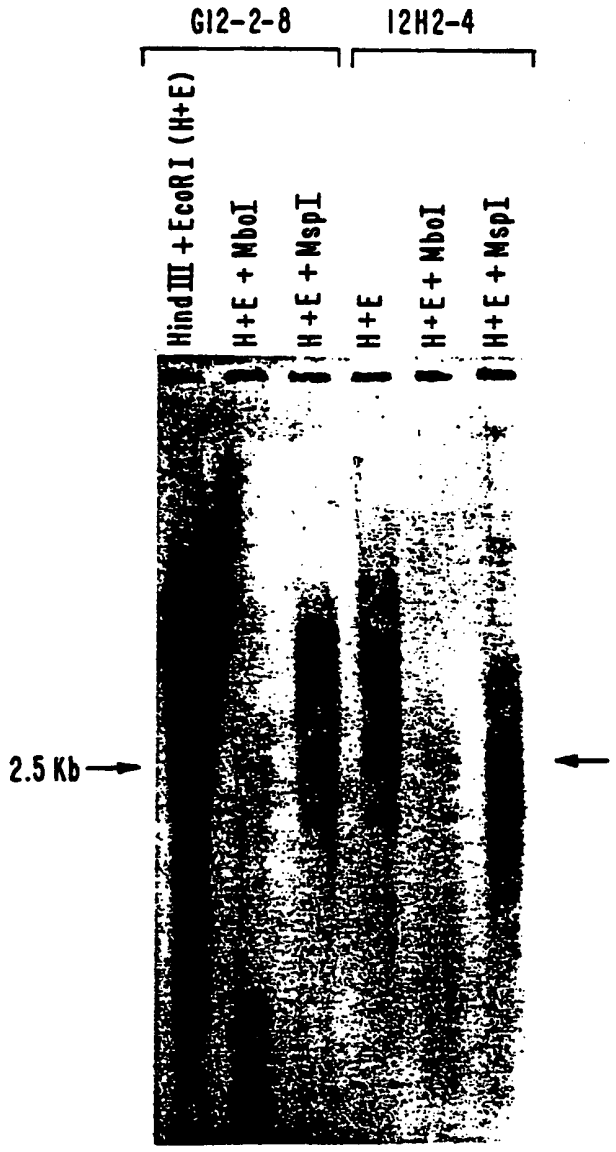

GI2-2-8
12H2-4
HindIII + EcoRI (H+E)
H+E + MboI
H+E + MspI
H+E
H+E + MboI
H+E + MspI
2.5 Kb

FIG. 15B

EXTRACTS

#4 #6

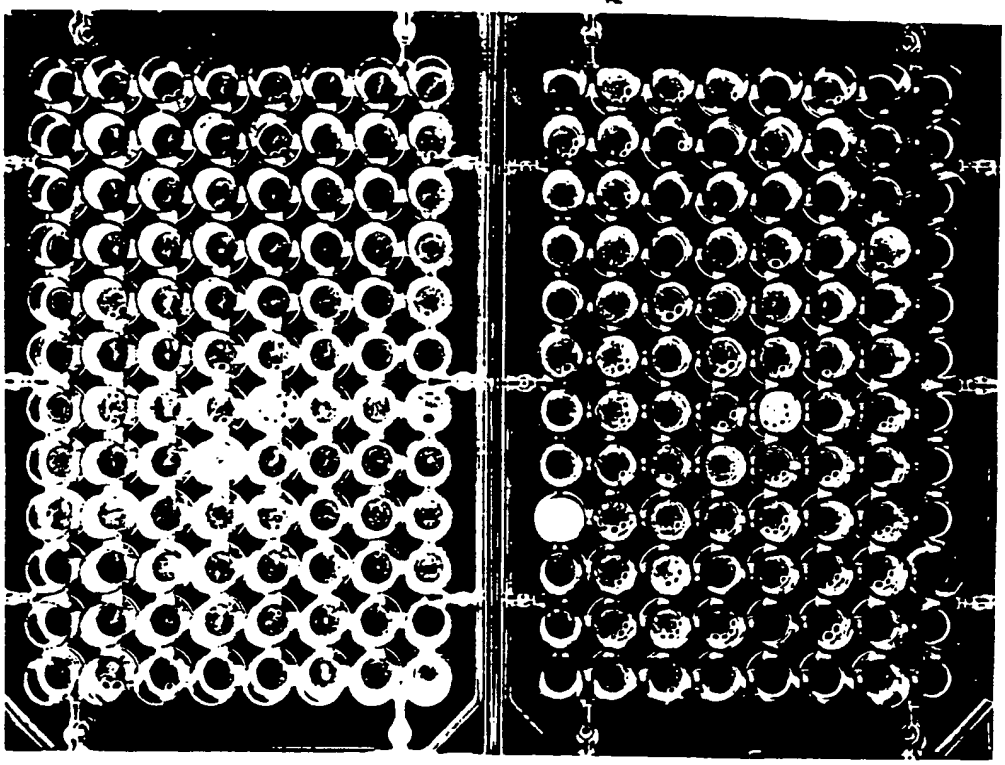
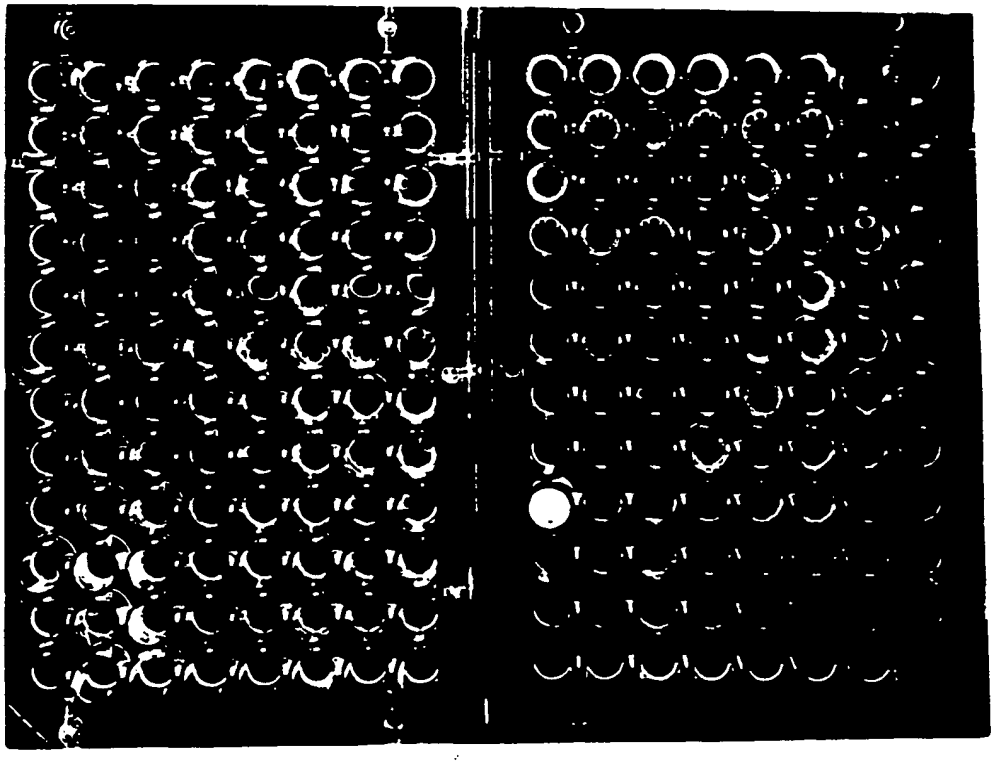


FIG. 15A

MEDIA



#5 #6

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/03192

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 12 N 15/82, A 01 H 5/00, A 01 H 5/10, C 12 P 21/02

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C 12 N, A 01 H, C 12 P

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Proc. Natl. Acad. Sci. USA, volume 83, February 1986, Y. Ohta: "High-efficiency genetic transformation of maize by a mixture of pollen and exogenous DNA", pages 715-719 see the whole document --	9,10,15, 16-20
X	The EMBO Journal, volume 6, no. 12, 1987, IRL Press Ltd, (Oxford, GB), V. Colot et al.: "Localization of sequences in wheat endosperm protein genes which confer tissue-specific expression in tobacco", pages 3559-3564 see the whole document --	21
X	EP, A, 0257472 (MAX-PLANCK-GESELLSCHAFT ZUR FÖRDERUNG DER WISSENSCHAFTEN e.V) 2 March 1988 see the whole document	9,10,15, 16-20
Y	-- ./.	1,4,5

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
10th November 1989	13. 12. 89
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	T.K. WILLIS

International Application No. PCT/US 89/03192

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Journal of Cellular Biochemistry, Suppl. 12C, 1988, UCLA Symposia on Molecular & Cellular Biology, Abstract, 17th Annual Meetings, 28 February - 10 April 1988, Alan R. Liss, Inc., (New York, US), A.K. Huttly et al.: "GA regulated expression from a wheat α-amylase promoter in oat aleurone protoplasts", page 207 see abstract L 600 cited in the application	1,4,5
P,X	EP, A, 0299552 (SOLVAY) 18 January 1989 see example II	1,5,9,10, 15,17,18, 20,21,23
P,X	EP, A, 0319353 (PLANT GENETIC SYSTEMS) 7 June 1989 see page 7, lines 39-40; claim 1	21
A	EP, A, 0270356 (AGRACETUS) 8 June 1988 see column 12, line 45 - column 13, line 6	1-23
A	WO, A, 83/01176 (INTERNATIONAL PLANT RESEARCH INSTITUTE) 14 April 1983	
A	WO, A, 87/00865 (CALGENE, INC.) 12 February 1987 see page 4, lines 12-16; claim 1	21-23

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 8903192 SA 30286

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 06/12/89
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0257472	02-03-88	JP-A- 63123325	27-05-88
EP-A- 0299552	18-01-89	NL-A- 8701450	16-01-89
EP-A- 0319353	07-06-89	AU-A- 2811889	23-05-89
		WO-A- 8903887	05-05-89
		EP-A- 0318341	31-05-89
EP-A- 0270356	08-06-88	AU-A- 8186387	09-06-88
		JP-A- 63258525	26-10-88
WO-A- 8301176	14-04-83	AU-A- 9054882	27-04-83
		EP-A- 0090033	05-10-83
WO-A- 8700865	12-02-87	AU-A- 6196686	05-03-87
		EP-A- 0233915	02-09-87
		JP-T- 63500425	18-02-88